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THE MECHANISM OF CAFFEINE-ENHANCED GLUCOSE STIMULATION OF LIVER GLYCOGEN SYNTHASE PHOSPHATASE ACTIVITY

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Abstract—Our report that glucose within its physiological range stimulates glycogen synthase phosphatase activity, provided an appropriate second effector is present, has been expanded The nature of the stimulatory process, particularly the roles of glucose, and of caffeine which represents the potential second effectors, has been studied. Glucose and caffeine stimulated synthase phosphatase activity in a synergistic manner With 0 5 mM caffeine the $A_{0.5}$ for glucose was 11 mM (from 27 mM), whereas in the presence of 30 mM glucose the $A_{0.5}$ for caffeine was 0 06 mM (from 0 7 mM) At 10 mM glucose the $A_{0.5}$ for caffeine was 0 1 mM Glucose stimulation remained non-cooperative, unaffected by the presence of caffeine, whereas the cooperative stimulation of caffeine was unaffected by glucose Some slight stimulation of synthase activity was observed with caffeine and with glucose over a wide concentration range However, they did not act synergistically to influence the measurement of synthase activity Glucose-6-phosphate, which also stimulates synthase phosphatase activity, acted independently, not synergistically with caffeine. All the methylxanthines were tested as potential second effectors in an effort to discover the essential structural elements of the agent All dimethylxanthines, 3- and 7-methylxanthine and 1-methyl-3-isobutylxanthine enhanced glucose stimulation but none of them alone was stimulatory. Judged from the half-maximal concentrations, in the presence of 10 mM glucose, caffeine was the most potent second effector by a significant margin The maximum velocity was also greatest with caffeine, whereas that with other methylxanthines was generally lower, and varied 1-Methylxanthine with increased concentration was slightly inhibitory even in the presence of 10 mM glucose Xanthine (0 5 mM), itself, strongly inhibited synthase phosphatase activity, an effect not influenced by glucose. Xanthine did not influence the measurement of synthase or phosphorylase phosphatase activity with or without glucose In general, conditions of methylxanthine-enhanced, glucose stimulation of synthase phosphatase and phosphorylase phosphatase activities differed markedly, confirming that separate, distinct mechanisms are involved

The rate of liver glycogen synthesis depends upon the proportion of glycogen synthase (EC 2.4.1.11) in the active form. This enzyme catalyzes the rate-limiting step in the pathway by which glucose is incorporated into glycogen. Activation of glycogen synthase is catalyzed by glycogen synthase phosphatase (EC 3.1.3.16). It is this reaction which must respond to agents that promote glycogen synthesis *in vivo*, although protein kinase activities may be simultaneously regulated. A rise in liver glucose concentrations can signal the activation of glycogen synthase [1–3]. However, the mechanism by which this is accomplished has been the subject of an intense debate.

Recently, we provided evidence that direct stimulation of the synthase phosphatase reaction by glucose [4, 5] may be one of the ways glucose promotes glycogen synthesis. Synthase phosphatase activity in a glycogen particle preparation could be more than doubled upon addition of glucose [4], but the concentration of glucose required greatly exceeded the upper limits of the normal physiological range Subsequently, caffeine was shown to stimulate synthase phosphatase activity [5] Remarkably, the presence of caffeine permitted glucose at a physiological concentration (10 mM) to stimulate the synthase phosphatase reaction almost 2-fold. This indicated that caffeine may act synergistically with glucose [5]

Glucose and caffeine are known to stabilize the "T" conformer of phosphorylase *a* and synergistically inhibit the enzyme in a competitive, non-exclusive manner [6]. The stabilized conformer is presumably a better phosphorylase phosphatase substrate [7–10], thus accounting for the observed increased phosphorylase phosphatase rate [4, 5, 11] when these ligands are present We, therefore, were interested in better characterizing the effect of caffeine on glucose stimulation of glycogen particle synthase phosphatase activity. We were particularly interested in determining the $A_{0.5}$ for glucose stimulation of synthase phosphatase activity in the presence of caffeine and the possible effect of glucose on the $A_{0.5}$ for caffeine stimulation of the activity. It was also important to establish that the observed effects of glucose and caffeine did not result from changes in the assay for synthase. Finally, to study the structural requirements of the second effector, the specificity of caffeine as a synergistic partner with glucose was determined by substituting various methylxanthines for caffeine This is part of an ongoing study of the mechanism(s) by which ingested carbohydrates stimulate glycogen synthesis in liver Phosphorylase

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phosphatase activity was determined simultaneously for comparative purposes

MATERIALS AND METHODS

Caffeine, theophylline, theobromine, xanthine, and 1,7-dimethylxanthine were obtained from the Sigma Chemical Co., St Louis, MO 1-Methylxanthine and 7-methylxanthine were obtained from the Chemical Dynamics Corp , Plainfield, NJ. 3-Methylxanthine was obtained from the Aldrich Chemical Co., Milwaukee, WI All other reagent chemicals were of the highest quality available

Male rats (Biolab, White Bear Lake, MN) weighing 150–200 g were anesthesized with Seconal (50 mg/kg, i p) and allowed a quiet period of at least 15 min Animals were then injected with glucagon (100 μg/kg, i v). Livers were rapidly removed 15 sec after glucagon administration and homogenized in ice-cold 50 mM imidazole, pH 7 0, with 250 mM sucrose (1 to 2, w/v). The homogenate was centrifuged at 8000 *g* for 10 min. The supernatant fraction was centrifuged at 104,000 *g* for 30 min to obtain the glycogen pellet. The pellet, washed to remove the microsomal layer, was suspended in one-half the volume of supernatant using 50 mM imidazole, pH 7.0

Synthase phosphatase and phosphorylase phosphatase assays were conducted simultaneously as previously described [4] Reaction mixtures contained glucose and xanthine or a xanthine derivative as indicated for each experiment Synthase phosphatase activity is expressed as the number of units of synthase I formed per gram wet weight generated per minute Phosphorylase phosphatase activity is expressed as the decrease in units of phosphorylase *a* per milliliter of glycogen particle suspension per minute. One unit of either synthase or phosphorylase is the amount of enzyme which incorporates 1 μmole of glucose into glycogen in 1 min at 30°

RESULTS

Glucose and caffeine effects on liver synthase phosphatase activity In the presence of a less than saturating concentration of caffeine (0 5 mM) [11], increased synthase phosphatase activity was observed as the glucose concentration was increased (Fig 1A). The $A_{0.5}$ for glucose was approximately 11 mM This was a substantial reduction from the $A_{0.5}$ measured previously in the absence of caffeine (27 mM) [4] When a saturating concentration of caffeine was used (2 mM), a concentration-dependent stimulation by glucose was also observed, and the $A_{0.5}$ for glucose remained 10 mM (data not shown) With glucose at 30 mM, the $A_{0.5}$ for caffeine was approximately 0 06 mM (Fig. 1B) In the absence of glucose, we had previously determined the $A_{0.5}$ to be approximately 0.7 mM [11] When 10 mM glucose was present, the $A_{0.5}$ for caffeine was estimated to be 0 09 mM (Fig 2) The sigmoidal relationship between phosphatase activity and caffeine concentration observed in both the absence [4] and the presence of glucose suggests cooperative stimulation with respect to caffeine.

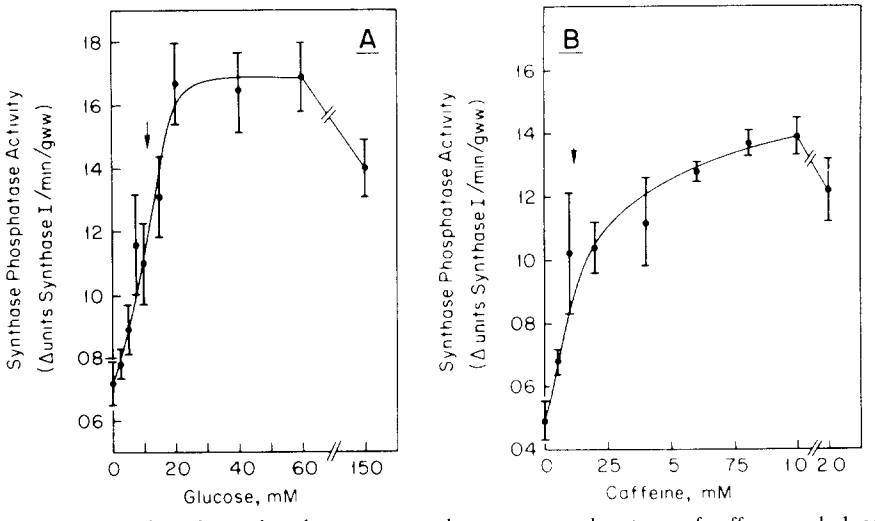

Fig 1 Stimulation of synthase phosphatase activity by various combinations of caffeine and glucose Liver glycogen particles were obtained from animals treated with glucagon as described in Materials and Methods These preparations were used in phosphatase reaction mixtures which contained, in addition, various combinations of caffeine and glucose Phosphatase assays were conducted at 25° Panel A shows the effect of a constant amount of caffeine (0 5 mM) and various amounts of glucose, as indicated, on synthase phosphatase activity Results are presented as the phosphatase activity expressed as the change in synthase I (Δ units/min/g wet wt) A unit is defined in Materials and Methods Phosphatase activity was estimated as the fastest rate in the incubation interval from 0 to 10 min The error bars represent the standard error and the arrow indicates the concentration where the velocity is half-maximal The initial activity ratio averaged 0 13 and total synthase was 0 92 units/g wet wt Panel B shows the effect of a constant amount of glucose (30 mM) and various amounts of caffeine, as indicated, on synthase phosphatase activity Conditions of the assay and data accumulation and reduction were the same as described for Panel A The average initial activity ratio was 0 22 and total synthase averaged 0 97 units/g wet wt Data presented in each panel are from four separate experiments

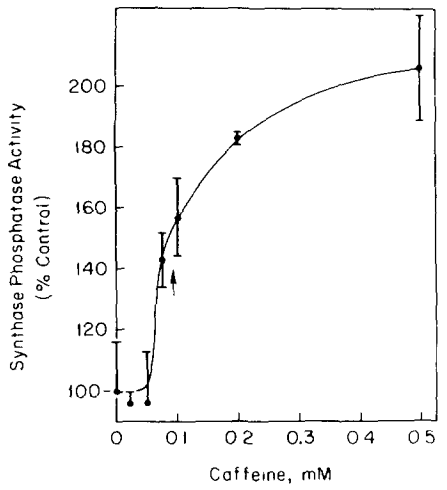

Fig. 2 Stimulation of synthase phosphatase activity by caffeine in the presence of 10 mM glucose Liver glycogen particles, obtained from glucagon-treated animals, were used to prepare phosphatase reaction mixtures containing 10 mM glucose and increasing concentrations of caffeine Mixtures were incubated at 25° and samples were withdrawn at appropriate intervals to analyze for synthase I and total synthase As in Fig 1, synthase phosphatase activity was estimated as the fastest rate in the incubation interval from 0 to 10 min Results are presented as the percentage of synthase phosphatase activity in a mixture containing 10 mM glucose. Each data point is the mean of three separate experiments ± S E M The arrow indicates the caffeine concentration where half-maximal activity was achieved ($A_{0.5}$) A data point not shown at 1 0 mM caffeine also showed stimulation 200% of control The initial activity ratio averaged 0 18 and total synthase averaged 0 95 units/g wet wt

In the present experiments, significant amounts of glucose and caffeine were transferred from the phosphatase assay mixture to the synthase assay. The apparent increased synthase phosphatase activity could result from specific stimulation of synthase activity, particularly by the mixture of caffeine and glucose. This was tested when amounts of glucose and caffeine, individually and in combination, equivalent to those transferred from phosphatase reaction mixtures, were added directly to synthase mixtures. The source of synthase was a resuspended glycogen particle preparation from glucagon-treated rats; portions of the resuspended particles were incubated at 30° for 0, 5 or 10 min to vary the ratio of synthase I to total synthase activity Measurement of total synthase was unaffected by any addition A moderate stimulation of synthase activity, measured in the absence of glucose-6-phosphate, was observed with 0.019 mM caffeine, equivalent to 0 5 mM in the phosphatase reaction mixture (Fig. 3). When the concentration was 0 076 mM, equivalent to 2 mM in the phosphatase reaction mixture, the amount of stimulation was not increased (data not shown). Similarly, synthase measured in the absence of glucose-6-phosphate was modestly stimulated by 0.37 mM glucose, equivalent to 10 mM glucose in the phosphatase reaction mixture, and the degree of stimulation was the same at various concentrations up to 2.96 mM (80 mM in the phosphatase mixture) The effect of the combinations of 0.019 mM caffeine and various glucose concentrations up to 2.96 mM was not greater than that of caffeine alone. The degree of stimulation in any case was not related to the relative proportion of synthase I. Synthase activity measured in the absence of glucose-6-phosphate was also only modestly stimulated by 0 5 mM caffeine and concentrations of glucose up to 80 mM, individually (Fig. 4), and stimulation by the combination did not exceed that of caffeine alone These concentrations would normally be sufficient to cause substantial conformational changes in phosphorylase *a* reflected as a decrease in its catalytic capabilities [6]. The stimulatory effects of glucose and caffeine as they influence synthase phosphatase activity could involve direct binding to synthase phosphatase However, if they interact with synthase, such an interaction has little effect on its catalytic capabilities

Effect of caffeine on glucose-6-phosphate stimulation of synthase phosphatase activity Glucose-6-phosphate stimulates glycogen particle-associated synthase phosphatase activity [11] The synthase phosphatase activity was increased to a greater extent when both glucose-6-phosphatase and caffeine were present. However, whether this was due to additive, independent effects or to a synergistic interaction between the two effectors, as was true for glucose and caffeine, was not determined. Therefore, synthase phosphatase activity was measured in the presence of 2 mM caffeine and increasing concentrations of glucose-6-phosphate (Fig 5). The approximate $A_{0.5}$ for glucose-6-phosphate was 0.12 mM. This was nearly the same as determined previously in the absence of caffeine [11]. In each case, the maximum stimulation by glucose-6-phosphate was the same Thus, caffeine and glucose-6-phosphate stimulated synthase phosphatase activity independently.

Methylxanthine structure and the facilitation of glucose stimulation of synthase phosphatase activity. The respective effects of the several methylxanthines on systems which they influence are dictated by their structures [12–14]. In the present studies, methylxanthines were compared for their influences on glycogen synthase phosphatase activity and their abilities to facilitate glucose stimulation of the reaction.

The parent compound, xanthine, at 0 5 mM inhibited rather than stimulated the synthase phosphatase reaction, and glucose at 10 mM had no effect on the inhibition (Fig 6A). Neither 0 019 mM nor 0.5 mM xanthine influenced the measurement of synthase in the absence of glucose-6-phosphate (data not shown). In contrast, xanthine had only a slight effect on the phosphorylase phosphatase reaction and did not interfere with glucose stimulation of the reaction (Fig. 6B). Substitution of a methyl group partially (position 1) or completely (positions 3 and 7) relieved the inhibitory effects of xanthine (Table 1) At 0.5 mM, neither 3- nor 7-methylxanthine stimulated the synthase phosphatase reaction. However, both the 3- and 7-methylxanthines in the presence of 10 mM glucose enhanced the synthase phosphatase rate The effect of 1-methylxanthine persisted even in the presence of 10 mM glucose

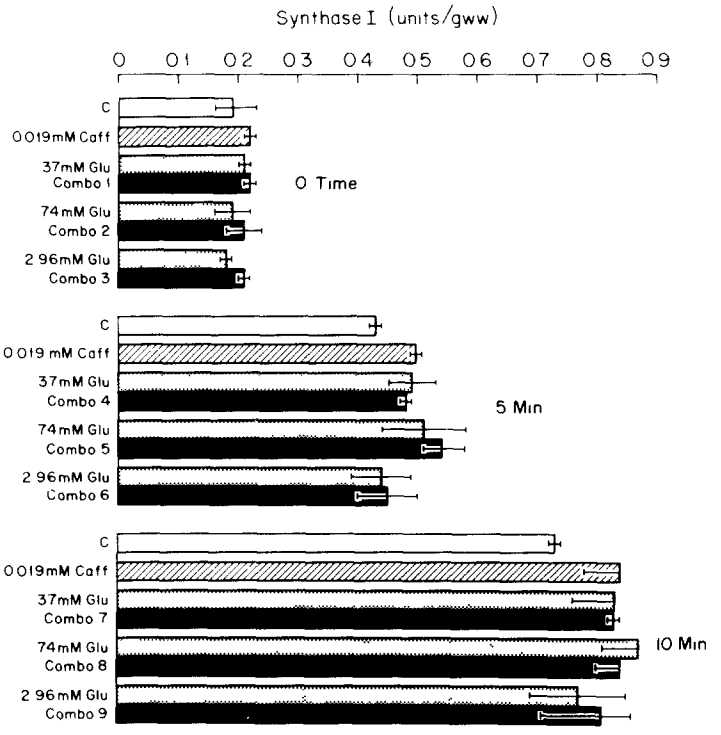

Fig 3 Effect of glucose and caffeine when present at low concentrations on the measurement of glycogen synthase Glycogen synthase present in glycogen particle preparations from glucagon-treated animals was measured in the presence of glucose with and without caffeine in amounts equivalent to those transferred from phosphatase reaction mixtures Following resuspension of the preparation in 50 mM imidazole, pH 7 0, portions of the resuspended particles were incubated for 5 or 10 min at 30° The O time control was kept on ice Then, 25 μl of each pellet suspension was diluted to a final volume of 225 μl in special synthase stopping reagents containing caffeine or glucose at various concentrations with and without caffeine In the experiments shown, the final concentrations were 1 11 2.22, and 8 88 mM glucose with or without 0 057 mM caffeine These are concentrations which would be obtained with the transfer and subsequent dilution of 25 μl of phosphatase incubation mixture had the original concentration been 10, 20 or 80 mM glucose, respectively, and that for caffeine 0 5 mM In the synthase assay, samples were diluted additionally to the final concentrations shown on the graph Particle suspensions diluted with stopping reagent without glucose and caffeine additions served as controls The results presented represent mean synthase activity (measured in the absence of glucose-6-phosphate) ± standard error for three separate determinations All combinations contained 0 019 mM caffeine (Caff) in addition to the amount of glucose (Glu) indicated

Alone, the monomethylxanthines had only a small stimulatory effect on phosphorylase phosphatase activity All three monomethylxanthines acted in an apparently synergistic manner with 10 mM glucose to enhance phosphorylase phosphatase activity Since only a single concentration of xanthine or xanthine derivative was tested, it is possible that a less than maximal effect was observed and that at higher concentrations the results might approach those of caffeine.

Unlike caffeine, none of the three dimethylxanthines (theophylline, theobromine and 1,7-dimethylxanthine) stimulated synthase phosphatase activity when present at 0 5 mM (Table 1) However, when added together with glucose which itself has little stimulatory affect on synthase phosphatase activity, there was a considerable stimulation of activity All three dimethylxanthines stimulated phosphorylase phosphatase activity, 1,7-dimethylxanthine was the most potent, being almost equivalent to caffeine Theophylline and theobromine further stimulated activity in the presence of glucose and were approximately equipotent in this regard In contrast, 1,7-dimethylxanthine had no apparent synergistic effect.

From the data in Table 1, caffeine and glucose seemingly did not act synergistically to enhance phosphorylase phosphatase activity. In fact, they did. The $A_{0\,5}$ for caffeine was determined to be about 0 08 mM for the phosphorylase phosphatase reaction (experiments not shown) Since caffeine and glucose stabilize the same conformer of phosphorylase [7], 0 5 mM caffeine stimulated phosphorylase maximally. As was observed, the addition of glucose would be expected to have no additional effect

1-Methyl-3-isobutylxanthine (MIX) binds with great affinity to both adenosine A_1 and A_2 receptors [12] and to phosphodiesterase [14] Because of its relative potency compared to other methylxanthines in binding to these sites, it was tested for its effects on both the synthase phosphatase and phosphorylase phosphatase reaction systems MIX, alone, did not

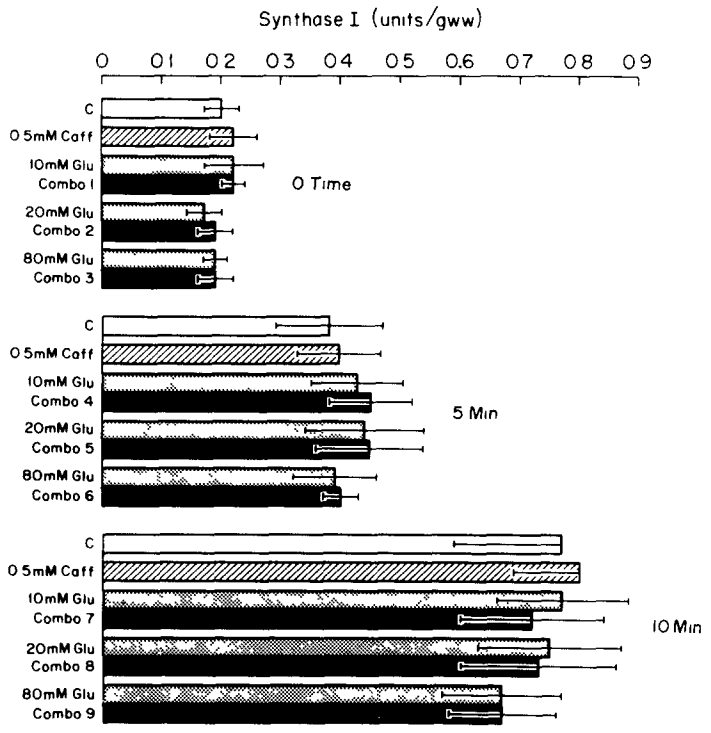

Fig 4 Effect of glucose and caffeine when present at high concentrations on the measurement of glycogen synthase This experiment was conducted as described in the legend for Fig 3 except that the final concentrations of glucose and caffeine in the synthase assay are those present in the phosphatase assay before dilution Therefore, in these experiments the final concentrations after dilution of resuspended particles with special stopping reagents were 30, 60 and 240 mM glucose with or without 1 5 mM caffeine When diluted in the synthase assay the concentrations were 10, 20 and 80 mM glucose, respectively, with or without 0 5 mM caffeine Resuspended particles diluted with stopping reagents without glucose and caffeine additions served as controls The results presented represent mean synthase activity (measured in the absence of glucose-6-phosphate) ± standard error for three separate determinations Combinations contain 0.5 mM caffeine (Caff) in addition to the indicated amount of glucose (Glu)

affect synthase phosphatase activity but a synergistic action with 10 mM glucose was apparent. Phosphorylase phosphatase was slightly stimulated by MIX but no synergistic action with glucose was observed

Those methylxanthines that enhanced glucose stimulation of synthase phosphatase activity were further characterized by estimating the $A_{0.5}$ for each in the presence of 10 mM glucose The concentration dependence of theophylline-enhanced stimulation is illustrated (Fig. 7) The $A_{0.5}$ for theophylline was approximately 0.7 mM, five to ten times greater than that for caffeine (Table 2). Maximum stimulation was slightly less than that for caffeine, and the stimulation was apparently cooperative with respect to theophylline. Sigmoidal-shaped curves of activity versus concentration also characterized the effects of other stimulatory methylxanthines (Table 2). Generally, the $A_{0.5}$ values for all methylxanthines except MIX were high, and the maximum stimulation was lower compared to caffeine

DISCUSSION

An examination of the experiments of Kasvinsky *et al.* [15] revealed that, when hepatocytes are incubated with 1 mM caffeine and 20 mM glucose, glycogen synthase activation occurs at a greater rate than with glucose alone. The rate of phosphorylase inactivation was, similarly, increased by the combination, compared to glucose only Synthase activation and phosphorylase inactivation were also simultaneous events. Yet, 1 mM caffeine had no apparent effect on either system, and 5 mM caffeine caused considerable phosphorylase activation. The latter effect is consistent with an elevation of cAMP and the activation of a cAMP-dependent protein kinase [16–18]. The cell-free preparation employed in the present studies has been useful because, as with hepatocytes, a combination of caffeine and a physiological concentration of glucose greatly enhanced the synthase phosphatase rate compared to that sustained by the individual effectors

The present observations provide strong support for the suggestion [5] that glucose and caffeine act synergistically to stimulate the synthase phosphatase reaction. These same effectors act synergistically to stimulate phosphorylase phosphatase activity [5] However, it is evident that the effects of these modifiers on the two reactions can be dissociated For example, only caffeine stimulated the two reactions itself Several other methylxanthines stimulated the

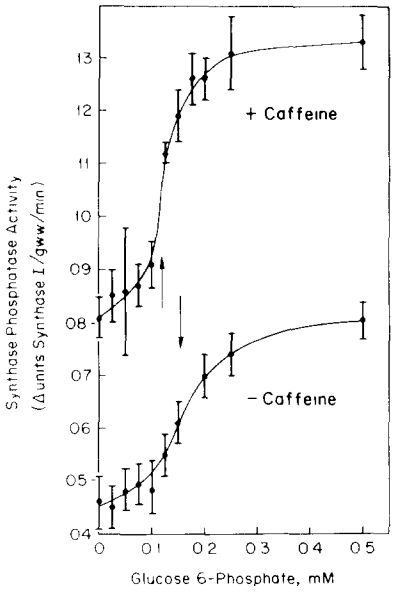

Fig 5 Effect of the combination of caffeine and glucose-6-phosphate on synthase phosphatase activity A control phosphatase reaction mixture prepared from liver glycogen particles obtained from a glucagon-treated animal contained 2 mM caffeine Other mixtures contained, in addition, various amounts of glucose-6-phosphate as indicated Results are expressed as synthase phosphatase activity (Δ units synthase I/min/g wet wt) measured as the fastest rate in the incubation interval from 0 to 10 min at 25° The half-maximal rate indicated by the arrow occurred when the glucose-6-phosphate concentration was 0 12 mM The error bars indicate the standard error for four separate determinations For comparison, the effect of glucose-6-phosphate, only, published previously [11], was reanalyzed and is shown in the figure In both instances, the maximal glucose-6-phosphate stimulation of the synthase phosphatase reaction was 170% of control

phosphorylase phosphatase reaction but not the synthase phosphatase reaction (see Table 1) On the other hand, xanthine inhibited the synthase phosphatase reaction but not the phosphorylase phosphatase reaction. Thus, with the distinct responses of the two phosphatase systems, the mechanisms of action of xanthine and its derivatives on the two systems must be different This difference was further seen when a standard concentration of each of the various methylxanthines was used to enhance glucose stimulation of the two phosphatase reactions Moreover, the measured half-maximal concentrations for glucose and caffeine stimulation of synthase phosphatase activity differ substantially from the kinetic constants determined for their interaction with phosphorylase *a* [6]

From the present results there is certainty that the effects of glucose and caffeine influence the phosphatase reaction rate and not the measurement of synthase I Unlike phosphorylase *a* which is inhibited by glucose and caffeine and is especially sensitive to the combination [6, 7], synthase activity was not influenced by these effectors This, of course, is no assurance that the loci for glucose and caffeine are not associated with synthase D rather than synthase phosphatase The relative potencies particularly of caffeine and theophylline, compared to MIX, suggest that the nature of their binding sites are different from the biologically important sites already characterized. Thus, for example, MIX binds with an affinity greatly exceeding that of caffeine and theophylline to the A_1 and A_2 adenosine receptors [12] and to phosphodiesterase [14]

In preliminary studies, phosphorylated derivatives of glucose were examined as possible substitutes for glucose in a synergistic interaction with caffeine Glucose-6-phosphate stimulation of synthase phosphatase activity was not influenced by caffeine as evidenced by the unchanged $A_{0.5}$ and the maximum stimulation Likewise, glucose-1-phosphate

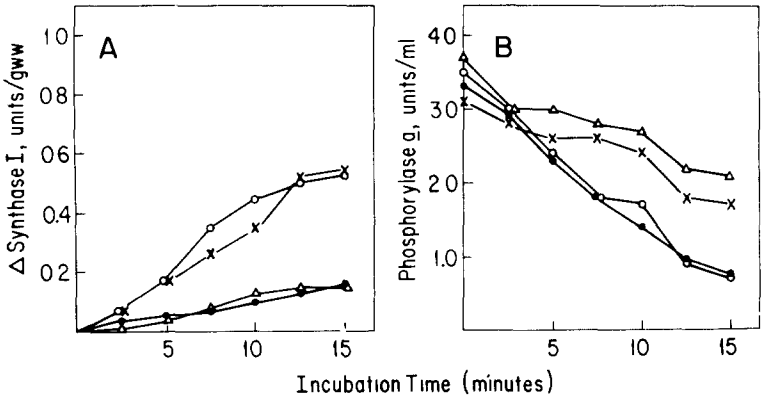

Fig 6 Effect of glucose and xanthine on synthase phosphatase and phosphorylase phosphatase activities Synthase phosphatase and phosphorylase phosphatase activities were measured in glycogen particle preparations from animals treated with glucagon Reaction mixtures were then incubated at 25° and contained, in addition to the glycogen particle, no addition (×—×) 10 mM glucose (○—○), 0 5 mM xanthine (△—△) or 0 5 mM xanthine and 10 mM glucose (●—●) Panel A shows the results of experiments measuring synthase phosphatase activity, while Panel B shows the phosphorylase phosphatase results Each experiment was repeated with four separate preparations, and the results are depicted as the mean of these experiments

Table 1 Effects of methylxanthines on synthase phosphatase and phosphorylase phosphatase activities in the presence and absence of glucose

Compound	N	% Control synthase phosphatase		% Control phosphorylase phosphatase +10 mM Glucose		
		−Glucose	+10 mM Glucose*	−Glucose	Predicted	Observed
(1) Caffeine‡	5	145 ± 16	245 ± 20	375 ± 66	668	450 ± 33
(2) Theophylline	3	106 ± 10	134 ± 4	293 ± 66	521	782 ± 77
(3) Theobromine	3	104 ± 15	136 ± 8	250 ± 33	445	696 ± 119
(4) 1,7-Dimethylxanthine	3	103 ± 10	134 ± 5	373 ± 3	663	633 ± 73
(5) 7-Methylxanthine	3	100 ± 14	150 ± 10	114 ± 21	203	436 ± 93
(6) 3-Methylxanthine	4	106 ± 7	160 ± 11	145 ± 15	258	465 ± 40
(7) 1-Methylxanthine	3	62 ± 10	72 ± 10	129 ± 7	230	464 ± 72
(8) 1-Methyl-3-isobutylxanthine	3	100 ± 15	180 ± 13	121 ± 10	102	191 ± 21
(9) Xanthine	3	27 ± 3	24 ± 3	91 ± 18	215	245 ± 27

* Results are compared to 10 mM glucose controls except in the case of caffeine where the control was 0 5 mM caffeine

† The predicted value is the stimulation expected if, in combination, the xanthine and glucose acted independently The observed value is the actual stimulation relative to the control in the presence of 10 mM glucose and a 0 5 mM concentration of the xanthine Except for the case of caffeine, if the observed value exceeded the predicted value, there is a strong suggestion of synergism Since the half-maximal concentration for caffeine stimulation of the phosphorylase phosphatase reaction was 0 08 mM (unpublished observation) with near maximum stimulation at 0 5 mM, glucose should not be expected to have an additional effect

‡ All xanthines were present at 0.5 mM

inhibited the synthase phosphatase reaction, an effect not relieved by caffeine (data not shown). In a physiological sense, glucose-6-phosphate, which is stimulatory in its normal concentration range, does not need a second effector to improve its binding affinity and to improve its effectiveness. On the other hand, glucose and certain analogues including fructose apparently require such an effector if they are to function in their normal physiological range.

The present results do not give a clear picture of the structural requirements for the second effector that enhances the ability of glucose to stimulate the synthase phosphatase and phosphorylase phosphatase reactions. All the dimethylxanthines and both the 3- and 7-methylxanthines enhanced glucose stimulation of synthase phosphatase activity with a resultant maximum stimulation less than that achieved with caffeine. However, caffeine stimulated the reaction with an affinity judged by the $A_{0.5}$ of almost an order of magnitude greater than that of other effective methylxanthines With regard to both the $A_{0.5}$ and maximum stimulation, caffeine and MIX may be equivalent. Contrasting these results, the methylxanthines showed a different pattern of effectiveness with respect to glucose-enhanced phosphorylase phosphatase activity although, again, at a standard concentration caffeine was by far the most effective

It seems clear from the evidence presented that glucose and caffeine act to stimulate the synthase phosphatase and phosphorylase phosphatase reactions in distinctly different, synergistic modes. If caffeine and glucose synergism reflects events of physiological importance, there are a number of questions to be answered Kasvinsky *et al* [15] showed that a crystal of muscle phosphorylase *a* has the nucleoside site occupied after soaking in a neutralized, perchloric acid extract of liver from a rat injected with glucose plus insulin but not in a similar extract from a non-injected control. Our work indicates that adenosine, a constituent of liver [19, 20], enhances glucose stimulation of synthase phosphatase activity The potential importance of the nucleoside site on phosphorylase *a* and an equivalent site in the synthase phosphatase system to enhance glucose regulatory effects makes identification of the natural ligand(s) a high priority. It is also important to determine whether the compound is constitutive or whether its concentration rises in response to an external signal What would the external signal be? It could be glucose, itself, or insulin might be involved.

Finally, the question arises as to the possible importance of the synergism between glucose and a second effector represented in these studies by caffeine Kasvinsky *et al* [6] discussed the relationship with respect to phosphorylase *a* and its inactivation by phosphorylase phosphatase At its presumed physiological concentration, AMP suppresses the effects of glucose and stabilizes a form of phosphorylase *a* which is catalytically active Contrariwise, glucose alone stabilizes a form of phosphorylase *a* which is inhibited and is a favored phosphorylase phosphatase substrate Only in the presence of caffeine are physiological concentrations of glucose able to overcome the powerful effects

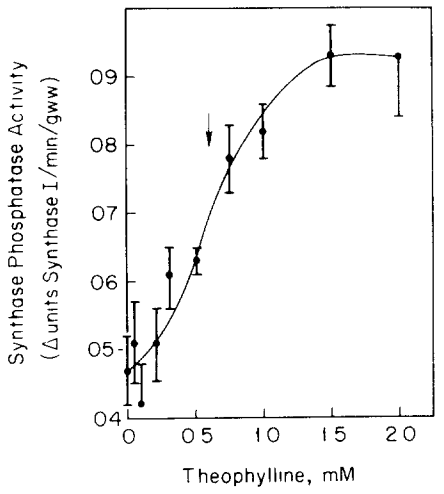

Fig 7 Stimulation of synthase phosphatase activity by 10 mM glucose in the presence of various concentrations of theophylline Glucagon-treated animals were the source of liver used to prepare glycogen particle preparations Phosphatase reaction mixtures contained, in addition to the particle preparation, 10 mM glucose with increasing concentrations of theophylline, as indicated The control contained only 10 mM glucose As in Fig 1, mixtures were incubated at 25° with samples withdrawn at appropriate intervals for the measurement of synthase I and total synthase Phosphatase activity was estimated as the fastest rate in the incubation interval from 0 to 10 min Activity is expressed as Δ units of synthase I/min/g wet wt The arrow indicates the half-maximal concentration of theophylline The initial activity ratio for the three separate experiments averaged 0 18 and total synthase averaged 0 95 units/g wet wt Data points are shown with standard error bars

of AMP These observations, and in particular the demonstration of the so-called nucleoside site, led the authors to speculate that glucose may not be the sole regulator of glucose homeostasis in liver.

We have reached a similar conclusion with respect to the synthase phosphatase reaction. Although physiological concentrations of glucose are able to overcome the powerful inhibitory effects of ATP-Mg^{2+} on the synthase phosphatase reaction (manuscript in preparation), stimulation of the reaction beyond the basal level requires glucose concentrations that exceed the physiological range When a site in the synthase phosphatase system equivalent to the nucleoside site on phosphorylase *a* is occupied by an appropriate effector, stimulation of the synthase phosphatase activity by physiological concentrations of glucose is possible. Stimulation to more than double the basal level and several times that when ATP-Mg^{2+} is present occurs in direct proportion to the glucose concentration Thus, although mechanisms may differ, the role of the second effector in both the synthase phosphatase and phosphorylase phosphatase reactions is to make physiological concentrations of glucose more effective in stimulating each of these reactions. Thus, glycogenolysis is reduced by inactivating phosphorylase *a* and glycogen synthesis is increased by activating glycogen synthase, by events which need not be sequential but can be simultaneous

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Table 2 Comparison of methylxanthines that enhance glucose stimulation of liver glycogen synthase phosphatase activity

Methylxanthine	N	$A_{0.5}$*	Maximum stimulation % of Control†	Maximum stimulation Concn (mM)
(1) Caffeine	4	0 1	219	0 5
(2) Theophylline	3	0 7	195	2 0
(3) Theobromine	3	0 5	170	1 0
(4) 1,7-Dimethylxanthine	3	0 4	175	2 0
(5) 7-Methylxanthine	3	0 5	170	1 5
(6) 3-Methylxanthine	3	0 3	180	1 0
(7) 1-Methyl-3-isobutylxanthine	3	0 1	201	1 0

* Determined in the presence of 10 mM glucose
† In each case except for caffeine, the control was 10 mM glucose For caffeine, the control was 0 5 mM caffeine

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