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EFFECT OF XANTHINES AND SOME DERIVATIVES ON PROTEIN METABOLISM IN ISOLATED PARENCHYMAL RAT LIVER CELLS

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Abstract—(1) Isolated parenchymal rat liver cells were incubated in Krebs–Henseleit buffer containing 0.5% gelatine. (2) Xanthines (caffeine, theophylline, theobromine, paraxanthine) and some derivatives (IBMX, aminophylline, proxiphylline) all inhibited the incorporation of [^{14}C]valine into proteins in the medium and albumin secretion. (3) The incorporation into liver cell proteins was only significantly inhibited by IBMX, caffeine and theophylline. (4) Xanthine and metabolites of xanthines had no or only slight effect on the variables mentioned above. (5) The inhibitory effect of xanthines and their derivatives on protein metabolism could not be explained by an increased level of cyclic AMP, a decreased secretion of proteins from the liver cells, an inhibition of amino acid transport, an increased degradation of proteins or an effect at the transcription level. (6) The results are compatible with an effect on protein synthesis on the translation level.

From earliest times, man has made beverages from plants containing xanthines. Further, these compounds and their derivatives have important pharmacological properties.

Only limited, and to some extent conflicting, information is available concerning the effect of xanthines on protein metabolism. Their effect on carbohydrate and lipid metabolism have earlier been studied to a greater extent [1, 2].

We have recently reported an inhibitory effect of caffeine, theophylline and aminophylline on the incorporation of [^{14}C]valine into proteins and on the secretion of albumin in isolated parenchymal rat liver cells [3]. In the present investigation we have studied the effect of xanthines and some of their derivatives and metabolites on the same variables as mentioned above. Further we have investigated whether the observed inhibitory effect of xanthines and some of their derivatives on protein metabolism involves cyclic AMP, protein secretion, amino acid transport, protein degradation or transcription.

MATERIALS AND METHODS

Preparation and incubation of liver cells. Female Wistar rats fed *ad libitum* and weighing approximately 200 g were used. Cells were prepared as earlier described [4]. The cells were incubated in a medium consisting of Krebs–Henseleit buffer [5] containing glucose (15 mM), amino acids as in rat plasma [6] and 0.5% (w/v) gelatine [7]. Xanthines, derivatives or metabolites were added in a final concentration as stated in tables and figures. All cell suspensions were preincubated for 30 min. The incubations were then started by adding [^{14}C]valine (0.25 μCi) and incubation time was 120 min, unless otherwise stated.

Incorporation of [^{14}C]*valine and albumin secretion.* Incorporation of [^{14}C]valine into liver cell proteins (i.e. incorporation into proteins in the cell suspension minus incorporation into the medium) and into proteins in the medium and albumin secretion (i.e. the immunologically measured increase in albumin concentration in the medium) were determined as earlier described [4, 7].

Cyclic AMP. Incubations were started by adding IBMX, theophylline or glucagon. Extraction of cyclic AMP from the cell suspension was performed as earlier described [8]. Cyclic AMP was measured by the protein-binding radioassay method [9–11].

Intracellular albumin-like material. In some experiments the total amount of intracellular albumin-like material (albumin and proalbumin according to Judah *et al.* [12] and Russell and Geller [13]) was determined. 750 μl cell suspension was layered over 1500 μl dibutylphthalate and centrifuged for 15 sec. (approximately 4000 $g \times$ min) The isolated cells were suspended in 150 μl water, sonicated [7] and analyzed for albumin as above. No correction was made for extracellular albumin, as preliminary experiments with [^{14}C]inulin and [^{14}C]sucrose showed that only 2–3 per cent of albumin in the pellet was contaminant from the medium.

Amino acid transport. The incubations were started by adding [^{14}C]cycloleucine in a final concentration of 0.2×10^{-3} M and derivatives of xanthines (1×10^{-3} M) or solvent. At times as stated 150 μl cell suspension was layered over 1000 μl dibutylphthalate (density: 1.05) and centrifuged as above, by which cells and medium were separated. The isolated cells were suspended in 150 μl, sonicated and counted for radioactivity. Correction for extracellular [^{14}C]leucine was performed by measuring the [^{14}C]inulin space.

Degradation of protein. Liver cells were incubated in standard medium as mentioned above, except that amino acids were omitted. 0.75 μCi [^{14}C]valine was added after 30 min of preincubation and the cells were further incubated for 120 min. At that time the cells were washed twice in incubation medium containing amino acids ten times the concentration of the standard medium. Cells were resuspended in the same medium containing derivatives of xanthines in a final concentra-

tion as indicated. The liberation of non-protein bound [^{14}C]valine was followed as a function of time. At intervals as stated 100 μl suspension was precipitated with 100 μl 10% TCA (w/v), centrifuged and finally the radioactivity in the supernatant was determined.

Effect of actinomycin D. The incubations were started by adding actinomycin D (5 μg/ml cell suspension) or both actinomycin D and derivatives of xanthines (10^{-3} M). Other details were as above.

Chemicals. (1) *Xanthines*: Caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) were from Sigma, St. Louis, U.S.A. Paraxanthine (1,7-dimethylxanthine) and theobromine (3,7-dimethylxanthine) were from Fluka, Buchs, Switzerland and Pfaltz & Bauer, CT, U.S.A. (2) *Derivatives of xanthines*: Aminophylline (theophylline ethylenediamine) was from Sigma, St. Louis, U.S.A. 3-isobutyl-1-methylxanthine (IBMX) was from Aldrich, Milwaukee, U.S.A. and proxiphylline (7[2-hydroxypropyl]-1,3-dimethylxanthine) was a gift from Pharmacia, Hillerød, Denmark. (3) *Metabolites*: 1-Methylxanthine, 3-methylxanthine, 7-methylxanthine, 3-methyluric acid and 7-methyluric acid were from Fluka, Buchs, Switzerland. 1-Methyluric acid and 1,3-dimethyluric acid were from Adams, IL, U.S.A. (4) *Others*: Xanthine was from Fluka, Buchs, Switzerland. Actinomycin D was from Sigma, St. Louis, U.S.A. Dibutylphthalate was from Merck, Schuchardt, W. Germany. L-[U-^{14}C]valine and [^{14}C]cycloleucine was from the Radiochemical Centre, Amersham, England. Other chemicals and materials were as earlier described [7].

Statistics. All values are expressed as mean ± S.E.M. The significance of the difference between means is determined by the paired-data *t* test.

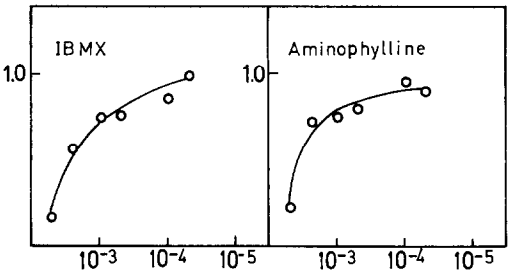

Fig. 1. Effect of IBMX and aminophylline on the incorporation of [^{14}C]valine into cell proteins in isolated parenchymal rat liver cells. Incubation time was 120 min. Abscissa: concentration (M). Ordinate: fractional incorporation of control.

RESULTS AND DISCUSSION

Effect of xanthine, xanthines, their derivatives and metabolites on protein metabolism. Addition of xanthine (10^{-3} M) had no effect on the incorporation of [^{14}C]valine into cell proteins and proteins in the medium or at the secretion of albumin (Table 1). Only theophylline, caffeine and IBMX (Table 1) at a concentration of 10^{-3} M had a significant inhibitory effect on the incorporation of [^{14}C]valine into cell proteins (9, 11 and 27 per cent, respectively). In contrast, all xanthines and derivatives significantly decreased the incorporation into proteins in the medium (14–62 per cent). The secretion of albumin was decreased in a similar manner, the inhibition being 26–68 per cent (Table 1).

With the concentrations used (5×10^{-5}–5×10^{-3} M) only IBMX and aminophylline showed dose–response between the concentration and the incorporation of [^{14}C]valine into cell proteins (Fig. 1). The

Table 1. Effect of xanthine, xanthines and derivatives (10^{-3} M) on the incorporation of [^{14}C]valine into cell proteins, proteins in the medium and on albumin secretion

	Incorp. of [^{14}C]valine into cell proteins		Incorp. of [^{14}C]valine into proteins in the medium		Albumin secretion	
	cpm × 10^{-3}/ml cells	Difference	cpm × 10^{-3}/ml cells	Difference	μg Albumin/ml cells	Difference
Control	1372 ± 147	—	863 ± 76	—	1117 ± 77	—
Xanthine	1321 ± 137	51 ± 34 n.s.	837 ± 93	26 ± 22 n.s.	1033 ± 64	84 ± 27 n.s.
Theophylline (1,3-dimethylxanthine)	1247 ± 109	125 ± 39*	720 ± 74	143 ± 30^{+}	734 ± 44	383 ± 37‡
Paraxanthine (1,7-dimethylxanthine)	1295 ± 125	77 ± 70 n.s.	716 ± 90	147 ± 35*	731 ± 82	386 ± 24§
Theobromine (3,7-dimethylxanthine)	1271 ± 126	101 ± 50 n.s.	745 ± 78	118 ± 22^{+}	823 ± 70	294 ± 35‡
Caffeine (1,3,7-trimethylxanthine)	1215 ± 126	157 ± 25	642 ± 73	221 ± 27‡	682 ± 83	435 ± 34‡
Aminophylline (theophylline ethylenediamine)	1274 ± 144	98 ± 41 n.s.	606 ± 65	257 ± 33‡	559 ± 50	558 ± 42§
IBMX (3-isobutyl-1-methylxanthine)	1002 ± 135	370 ± 28§	330 ± 45	553 ± 34§	356 ± 22	761 ± 56§
Proxiphylline (7-(2-hydroxypropyl)-1,3-dimethylxanthine)	1298 ± 132	74 ± 23 n.s.	713 ± 73	150 ± 12‡	885 ± 72	232 ± 17§

Statistical significance when compared with control: n.s. no statistical significance, * $P < 0.05$, $^{+}$ $P < 0.02$, ‡ $P < 0.01$, § $P < 0.001$.

Parenchymal rat liver cells were preincubated 30 min with the substances stated or solvent before [^{14}C]valine was added. The incubation time was 120 min. Figures are given as means ± S.E.M. of 4 experiments.

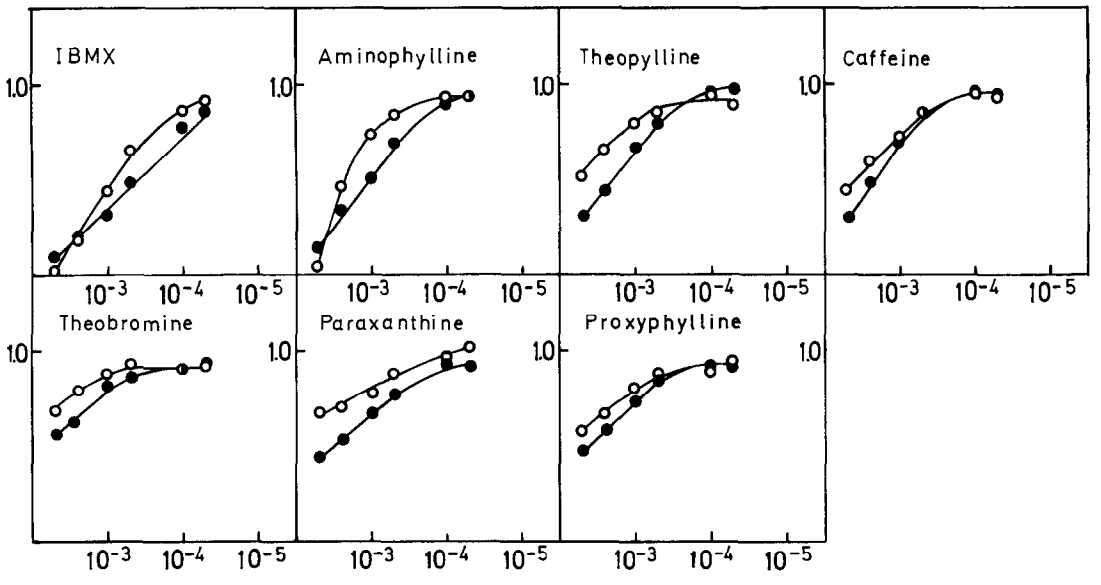

Fig. 2. Effect of IBMX, aminophylline, theophylline, caffeine, theobromine, paraxanthine and proxiphylline on the incorporation of [^{14}C]valine into proteins in the medium (○) and on albumin secretion (●). Details as in Fig. 1.

opposite dose–response relationship existed between all the xanthines investigated and the incorporation of [^{14}C]valine into proteins in the medium and the secretion of albumin (Fig. 2). The inhibition of the last mentioned variables was very similar. IBMX and aminophylline seemed to have an effect in a concentration of 10^{-4} M or lower. Xanthines and proxiphylline had an increasing inhibitory effect at concentrations higher than 10^{-4} M.

These results confirm and extend earlier observations that xanthines and their derivatives have an inhibitory effect on the incorporation of amino acids into proteins and on albumin secretion in isolated parenchymal liver cells [3]. It has earlier been demonstrated that xanthines *in vitro* have an inhibitory effect on amino acid incorporation into proteins in muscle [14], fat [15, 16], brain [17] and endocrine tissues [18–20].

The degradation of xanthines goes mainly through demethylation and oxidation before excretion in the urine. The liver is known to be the primary site of metabolism [21]. Only about 10 per cent of the xanthines is excreted unchanged [22]. As the *in vivo* half life of caffeine and theophylline in the rat has been reported to be between 1.5 and 6.5 hours [23–25], the metabolites could be active in the present study. However, the metabolites, as reported by Cornish and Christman in man [22], has in the present study no significant effect on the incorporation of [^{14}C]valine into liver cell proteins and proteins in the medium, when a concentration of 10^{-3} M was used (data not shown). Most of the metabolites had a slight but significant effect on albumin secretion (Table 2). The metabolism of theophylline in rat has earlier been shown to be similar to that of man [26].

Table 2. Effect of metabolites of xanthines on albumin secretion from isolated parenchymal rat liver cells

	Albumin secretion	
	μg Albumin/ml cells	Difference
Control	1117 ± 77	—
1-Methylxanthine	1025 ± 62	93 ± 23*
3-Methylxanthine	1084 ± 75	33 ± 6+
7-Methylxanthine	991 ± 53	126 ± 32*
1-Methyluric acid	1066 ± 86	51 ± 5+
3-Methyluric acid	1116 ± 71	1 ± 15 n.s.
7-Methyluric acid	1085 ± 62	32 ± 28 n.s.
1,3-Methyluric acid	1066 ± 72	51 + 15+

Statistical significance when compared with control: n.s. no statistical significance, * $P < 0.05$, + $P < 0.02$.
Experimental details as in Table 1.

In summary, xanthine and monomethylated xanthines have no or only a small effect on protein metabolism, while dimethylated (paraxanthine, theobromine and theophylline) and trimethylated (caffeine) xanthines have a significant inhibitory effect (Tables 1 and 2). Further, the oxidized mono- and dimethylated xanthines seem to have less inhibitory potency than their non-oxidized counterparts (Tables 1 and 2). The liver microsomal system, which have been shown to participate in the demethylation and oxidation of xanthines [21], seems to be active in detoxifying xanthines.

Aminophylline inhibited protein metabolism more than theophylline (Table 1). This could be due to ethylenediamine, which is known to be dissociated from aminophylline and to inhibit incorporation of [^{14}C]phenylalanine into proteins [27].

Effect of xanthines on cyclic AMP. Addition of xanthines or derivatives could theoretically increase the level of cyclic AMP through an inhibition of phosphodiesterase. Further the increased concentration of cyclic AMP after addition of glucagon to isolated liver cells correlates well with the inhibition of incorporation of [^{14}C]leucine and albumin secretion [7]. An increased

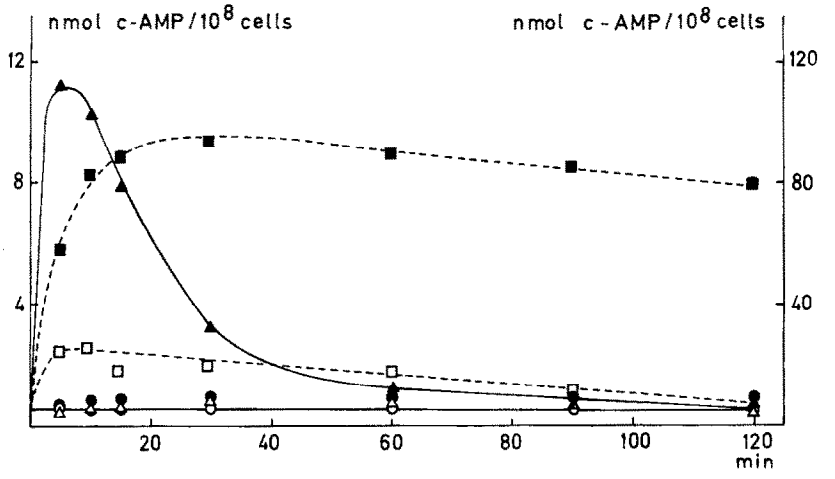

Fig. 3. Time course of cyclic AMP levels in parenchymal rat liver cell suspensions following xanthine derivatives glucagon or both. *Left ordinate*: ○—○ control; ●—● IBMX (10^{-3} M); △—△ theophylline (10^{-3} M); ▲—▲ glucagon (10^{-8} M). *Right ordinate*: □---□ theophylline (10^{-3} M) and glucagon (10^{-8} M); ■---■ IBMX (10^{-3} M) and glucagon (10^{-8} M). Each point is mean of 3 experiments.

level of cyclic AMP could be responsible for the impaired protein metabolism. The basal level of cyclic AMP was 0.54 ± 0.04 nmoles/10^8 cells ($n = 14$), which is in agreement with earlier findings [7]. The effect of IBMX and theophylline on the level of cyclic AMP is shown in Fig. 3. IBMX (10^{-3} M) or theophylline (10^{-3} M) did not augment the basal level. Glucagon (10^{-8} M) raised the concentration of cyclic AMP. The basal levels were reached after 60 min of incubation. Addition of glucagon and IBMX or theophylline raised the concentration of cyclic AMP further, and the basal level was not reached during the incubation time. These results are in agreement with earlier reports [28]. Our results do not support the hypothesis that xanthines regulates protein metabolism through an effect via cyclic AMP.

Effects of IBMX and aminophylline on intracellular albumin-like material. The inhibitory effect of xanthines and the derivatives was most pronounced on the proteins in the medium (Table 1). This could be due to an inhibition of the secretory process. As seen from Table 3, the derivatives investigated did not cause an accumulation of albumin-like material in the cells. On the contrary the amount decreased to 50–70 per cent of the control value at the end of the incubation.

A differentiated effect of xanthines inhibiting the metabolism of export proteins more than other proteins is in agreement with *in vivo* findings. A higher incidence of generalized edema and decrease of total plasma protein concentration in the offspring of rats receiving xanthines has been reported [29, 30]. No histological changes were found in the livers of the offspring of rabbits treated with caffeine [31].

Effects of xanthines on amino acid transport. The decreased incorporation of [^{14}C]valine into proteins could be due to a decreased transport of tracer into cell. Valine and the non-metabolizable amino acid, cycloleucine, are assumed to be transported by the Na^+-independent L-system of Christensen [32]. As seen from Fig. 4 the initial rate of influx is not significantly different whether IBMX or theophylline are present or not. Meanwhile, IBMX seems to increase the total uptake relative to the control.

Table 3. Effect of derivatives of xanthines on intracellular albumin-like material

	Intracellular albumin-like material μg Albumin/ml cells
Control	139 ± 21
IBMX	69 ± 13
Aminophylline	95 ± 9

Experimental details are given in Materials and Methods. Incubation time was 120 min. Figures are mean ± S.E.M. of 3 experiments.

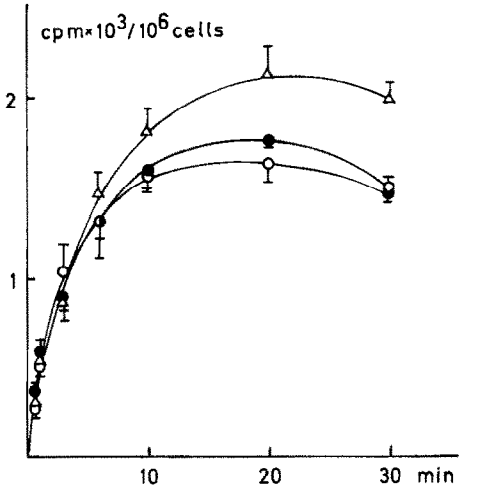

Fig. 4. Effect of IBMX and aminophylline on the influx of [^{14}C]cycloleucine into isolated parenchymal rat liver cells. ○ control, △ IBMX (10^{-3} M); ● aminophylline (10^{-3} M). Each point is mean ±S.E.M. of 4 experiments.

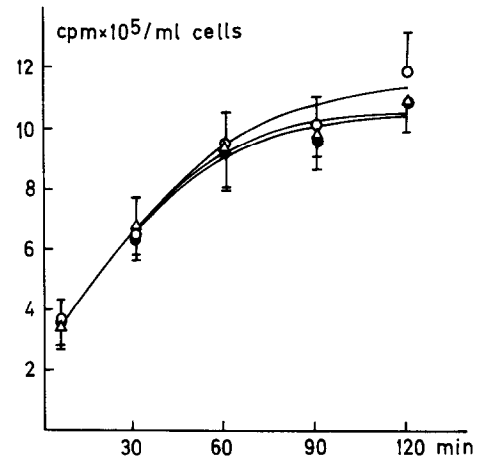

Fig. 5. Effect of derivatives of xanthines on the liberation of free [^{14}C]valine from pre-labelled isolated parenchymal rat liver cells. Experimental details as described in Materials and Methods. ○ control; ● IBMX (10^{-3} M); △ aminophylline (10^{-3} M). Each point is mean ± S.E.M. of 3 experiments.

This is in agreement with the earlier observations that theophylline does not increase the distribution ratio between the liver and plasma for non-metabolizable amino acid analogues transported by the Na^+-independent system [33]. Further, the transport of valine is very fast [34]. With the valine concentration used (0.2 mM) isotope equilibrium between the medium and the intracellular amino acid pool is obtained within a few minutes [34]. Thus a decreased incorporation of [^{14}C]valine is unlikely to be due to a diminished uptake of amino acids.

Effect of IBMX and aminophylline on protein degradation. It has earlier been demonstrated that [^{14}C]valine only to a small extent is metabolized in the liver [35]. Increase of the amount of free [^{14}C]valine liberated from pre-labelled liver cells would therefore be a measure of proteolysis. The time course of liberation of [^{14}C]valine from pre-labelled liver cells is shown in Fig. 5. Addition of IBMX or aminophylline (10^{-3} M) did not augment the liberation of [^{14}C]valine. An increased degradation seems not to be the reason for the decreased incorporation of [^{14}C]valine and albumin secretion, which is in accordance with results showing that theophylline (5×10^{-3} M) significantly decreased urea production in isolated liver cells (Gluud and Dich, unpublished observations).

Possible effect of xanthines on protein synthesis. From the excluded possible ways of affecting protein metabolism as tested above, xanthines could probably affect protein metabolism through an inhibition of protein synthesis. The inhibition could be either at the transcription or at the translation level.

Addition of actinomycin D, which inhibit RNA synthesis [36], gives rise to an inhibition of the incorporation of [^{14}C]valine into the proteins in the medium and to the secretion of albumin (controls, Table 4). The latter finding could be explained by the half-life of m-RNA for serum albumin being about 2–4 hr [37]. The pre-existing amount of m-RNA is not sufficient to maintain albumin synthesis in the experimental period.

If xanthines inhibit protein metabolism through an effect on transcription only, then no effect of xanthines on protein metabolism would be expected when actinomycin D is present. This is not the case. The percentual inhibition of xanthines on the measured variables was the same whether actinomycin D was present or not (Table 4). It is therefore concluded that protein metabolism can be regulated by xanthines at the translational level, either directly or indirectly.

The results does not rule out that xanthines could have an effect on RNA-synthesis. It has earlier been demonstrated that pentoxiphylline inhibits the incorporation into RNA and proteins to a similar extent and with a similar time course [17]. However, such an effect is not responsible for the inhibitory effect of xanthines on protein synthesis in the present study.

We have earlier stressed the opposite effect of xanthines on protein metabolism in cell and cell-free systems of the liver [3], and recently the same observa-

Table 4. Effect of actinomycin on the inhibition of IBMX and aminophylline on protein metabolism in parenchymal rat liver cells

	Incorporation of [^{14}C]valine into cell proteins		Incorporation of [^{14}C]valine into proteins in the medium		Albumin secretion	
	cpm × 10^{-3}/ml cells	% of control	cpm × 10^{-3}/ml cells	% of control	μg Albumin/ml cells	% of control
−Actinomycin						
Control	1260 ± 105	—	940 ± 67	—	1296 ± 67	—
IBMX (10^{-3} M)	745 ± 73	59 ± 1	326 ± 35	35 ± 1	428 ± 61	33 ± 3
Aminophylline (10^{-3} M)	1151 ± 83	92 ± 3	638 ± 47	68 ± 1	728 ± 66	56 ± 2
+Actinomycin (5 μg/ml cell suspension)						
Control	1131 ± 55	—	770 ± 64	—	877 ± 66	—
IBMX (10^{-3} M)	619 ± 87	55 ± 7	266 ± 33	34 ± 2	334 ± 34	38 ± 1
Aminophylline (10^{-3} M)	1020 ± 110	90 ± 5	548 ± 52	71 ± 2	597 ± 15	69 ± 4

The cells were preincubated 30 min before [^{14}C]valine, actinomycin, IBMX, aminophylline or solvent were added. Incubation time was 120 min. Figures are mean ± S.E.M. of 3 experiments.

tions have been obtained using the brain [17]. These results point to an indirect regulation of xanthines on protein metabolism.

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