Biochemical Pharmacology, Vol. 27, pp. 2675–2682
Pergamon Press Ltd. 1978. Printed in Great Britain.

CYCLIC AMP IN FRESHLY PREPARED THYMOCYTE SUSPENSIONS. EVIDENCE FOR STIMULATION BY ENDOGENOUS ADENOSINE

BERTIL B. FREDHOLM, GÖRAN SANDBERG and ULF ERNSTRÖM
Department of Pharmacology and Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden

(*Received* 6 *February* 1978; *accepted* 18 *April* 1978)

Abstract—Thymocyte suspensions were prepared from guinea pig thymus by gentle mincing and repeated washings. The mincing resulted in a ten-fold increase of the cyclic AMP level, most of the cyclic AMP being intracellular. The level remained high during the washing procedures. During incubation at 37° the level of intracellular cyclic AMP gradually fell by a temperature dependent process.

The cyclic AMP content was increased by non-methylxanthine phosphodiesterase inhibitors (papaverine, Ro 20-1724, ZK 62.711) but decreased in the presence of theophylline or isobutylmethylxanthine. Adenosine and phenylisopropyl adenosine acutely increased cyclic AMP by a theophylline inhibited mechanism. An increase was also obtained by inhibitors of adenosine uptake (dipyridamol, dilazep) and of adenosine deaminase (EHNA).

The results were compatible with a release of adenosine during the isolation of thymocytes, and with an adenosine-induced increase of intracellular cyclic AMP. This interpretation was supported by *in vivo* labelling of purine nucleotides with [^{3}H]adenine. A marked rapid fall of purine nucleotides was found during the preparation of the thymocytes and at the same time adenosine metabolites appeared in the medium.

A deficiency of adenosine deaminase (ADA, E.C. 3.5.4.4) has been found in children suffering from severe combined immunodeficiency [1–5]. A deficiency in purine nucleoside phosphorylase, which catalyzes the transformation of inosine to hypoxanthine was described in a child with isolated T-cell dysfunction [6]. The immune response of affected lymphocytes *in vitro* can be restored by addition of exogenous ADA, suggesting a causal link between immunodeficiency and depressed ADA activity [7].

Adenosine is mainly inactivated by deamination [8]. A deficiency in ADA is therefore likely to elevate adenosine and to decrease the appearance of the metabolites, inosine and hypoxanthine. This has also been reported in lymphocytes deficient in ADA [9]. High concentrations of adenosine are known to inhibit proliferation of normal lymphocytes [10, 11].

The suppression of lymphocyte function by adenosine is markedly potentiated by inhibitors of adenosine deaminase [12, 13]. Therefore one probable reason why ADA deficiency is associated with depressed T- and B-cell function is that adenosine is increased to toxic concentrations.

The mechanisms behind the depressing effect of adenosine on lymphocyte function is not clear. The induction of a state of pyrimidine starvation, at least partly due to the depletion of phosphoribosylpyrophosphate, has been described [14]. Another, and not necessarily unrelated possibility is that adenosine acts by elevating the cellular level of cyclic AMP. In several different cells, including lymphocytes, adenosine has been shown to elevate cyclic AMP levels [15–17]. An increased cyclic AMP content in lymphocytes is associated with a decreased cytolytic activity [16], decreased DNA synthesis [18] and decreased lymphocyte proliferation [19]. Cyclic AMP also inhibits the phosphoribosylpyrophosphate synthesis [20]. Thus, elevated cycle AMP may be one mechanism of cellular pyrimidine starvation.

Release of adenosine has been demonstrated from several different tissues and cell types [e.g. 21–23]. The possibility, therefore, exists that adenosine released from thymocytes and/or other cells suppresses lymphocyte activity, possibly via stimulation of cyclic AMP production. The effect of endogenous adenosine might be enhanced by a deficiency in ADA activity. A marked increase in cyclic AMP levels in a patient with ADA deficiency has been reported [24], in keeping with such a view. A specific regulatory role of adenosine and ADA in lymphoid tissues is also supported by the observation that ADA activity is highest in tissues with a high lymphocyte content [25] and that antigenic stimulation of lymph nodes leads to an increased ADA activity of the efferent lymphocytes [26].

In the present paper we report that during preparation of a thymocyte suspension from guinea pig thymus there is a parallel increase in purine release and in cell cyclic AMP content. Furthermore, the increase in cyclic AMP is potentiated by substances that inhibit adenosine inactivation and decreased by substances that antagonize adenosine. We interpret these results as further support for a role of endogenous adenosine in the regulation of thymocyte cyclic AMP levels. A preliminary account of some of these data has been given in abstract form [27].

MATERIAL AND METHODS

Preparation of thymus cell suspensions. Male outbred guinea pigs weighing 250–300 g were used as cell donors. After anaesthesia with 2.5 per cent pentobarbital sodium the two thymus lobes were excised and placed in cold (0°) buffer consisting of equal parts of Hank's balanced salt solution (BSS) and Dulbecco's phosphate buffered saline (PBS). They were then cut into small pieces on top of a nylon net and rinsed with cold buffer while stirred with a glass rod. The cell suspension obtained was freed of cell aggregates and tissue debris by filtration through fine meshed nylon nets. In some cases, the thymus was retained in the buffer and cut into small pieces which were repeatedly passed through a pasteur pipette. Aggregates were removed by sedimentation. In either case, the cells were washed twice in buffer, resuspended in medium (RPMI 1640 tissue culture medium from Flow labs supplemented with 2 μmol L-glutamine, 100 i.u. penicillin and 100 μg streptomycin per ml) and adjusted to 2×10^6 or 10^7 cells per ml. No serum was added. The preparation of the cell suspension was either carried out at room temperature (but keeping the temperature of the cells low by using cold buffers and medium) or in a few specified cases in a cold room at 4°.

Incubation. Amounts of 1 ml of the cell suspension were added to plastic tissue culture tubes (Falcon, 12 × 75 mm) and incubated at 37° (or in some cases at 4°) in 10 per cent CO_2 in air. Drugs were dissolved in water and sterilized by Millipore filtration. The drugs were added either to the buffer and medium used during the preparation procedure, at the start of incubation, or after various times of preincubation.

Determination of cAMP. For determination of total cAMP content, 250 μl samples were taken after various incubation periods after resuspension of the cultured cells. The samples were heated to 80° for 5 min, frozen and stored at −20° for 2–40 days until assay. No significant effect of storage on cyclic AMP levels was found.

In order to separate cellular and extracellular cyclic AMP part of the culture was filtered through a Millipore filter (0.22 μm pore size) and the cyclic AMP content in the filtrate and intact cell culture was compared. In other experiments the cultures were centrifuged (176 *g*, 10 min) and the cyclic AMP content measured in the supernatant and in the pellet after extraction with 0.4 M perchloric acid (PCA).

Cyclic AMP was also determined in homogenates of thymus tissue: One thymus lobe was rapidly excised and frozen in liquid propane (−196°), crushed and homogenized in 0.4 M PCA by an all glass homogenizer. The other lobe was taken out at room temperature, cut in small pieces (i.e. the initial steps in the preparation of the cultures) before homogenization in PCA.

PCA-extracts were centrifuged, and the supernatant neutralized with 50 mM Tris and sufficient 4 M KOH to bring the pH to 7.2–7.6. The other samples were used directly in the assay. The competitive binding assay of Brown *et al.* [28] was used with ^{3}H-cyclic AMP (27 Ci/mmol) from the Radiochemical Centre (Amersham) and binding protein prepared from bovine adrenal cortex. Five mM EDTA, 100 mM NaCl and 0.1 % bovine serum albumin was included in the assay buffer. Of the binding activity 90 ± 4 per cent was removed by treatment with cyclic nucleotide phosphodiesterase partly purified from beef heart (Boehringer, Mannhein). Serial dilutions of samples fell on the standard curve. Purification of cyclic AMP on Dowex 1 × 2 (Cl-form) gave results that were statistically indistinguishable from those obtained in unpurified samples. Thus, the authenticity of cyclic AMP was considered proven.

In vivo *labelling of thymocyte purine stores.* [^{3}H]-adenine (22 Ci/mmol, Radiochemical Centre, Amersham) was dissolved in physiological saline to a final concentration of 1 mCi/ml. 0.1 ml of this solution was injected intracardially in four animals. After 60 min the thymus lobes were dissected out. At various stages of the preparation of thymocytes samples were taken, rapidly frozen in liquid propane, homogenized in 0.4 M perchloric acid and frozen.

Separation of labelled purines. The extracts were centrifuged. The protein-free supernatant was neutralized with 4 M KOH and 1 M Tris-base. The entire neutralized sample (less an aliquot taken for determination of total radioactivity) was put on small columns (0.7 × 2.5 cm) of Dowex 1 × 8 (Cl^-). Nucleosides and bases were eluted with water, nucleotides with 0.01 M and 2 M HCl. An aliquot of each fraction were taken for assay of radioactivity, the remainder was lyophilized and taken up in a minimal quantity of water. The purines were then separated either on silica TLC according to Schimizu *et al.* [29] or on PEI-cellulose according to Böhme and Schultz [30]. Furthermore, some extracts were separated using high-pressure liquid chromatography as described by Nordström *et al.* [31]. Values are expressed as nCi/g tissue.

Drugs. Adenosine and isoprenaline hydrochloride were obtained from Sigma (St. Louis, USA), papaverine and theophylline (as the ethylendiamine-Theophyllamin^R) from ACO (Stockholm, Sweden), 3-isobutyl-1-methylxanthine from Aldrich Chemicals, adenosine deaminase (dialyzed twice against 1000 volumes of medium before use) and [N^6]phenylisopropyl adenosine (PIA) from Boehringer (Mannheim, GFR). Indomethacin was obtained from MSD (Rahway, N.Y., USA). Dilazep was a gift from Dr. Brock, Astawerke (Brackwede, GFR) and dipyridamol from Boehringer Ingelheim (GFR). The adenosine deaminase inhibitor erythro-9-(2 hydroxy-3-nonyl) adenine hydrochloride (EHNA) was obtained from Wellcome Research Laboratories (Research Triangle Park, USA). 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidon (ZK 62.711) was a gift of Dr. Kehr, Schering AG (Berlin, GFR) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidane (Ro 20-1724) was kindly supplied by Hoffman La Roche.

RESULTS

The content of cyclic AMP in thymus tissue frozen directly *in situ* averaged 0.9 nmol/lobe (0.886 ± 0.193, mean ± S.E. $n = 7$). The total number of lymphocytes per thymus was calculated by Ernström and Sandberg [32] to be 1.04×10^9. Thus, the cyclic AMP content of thymus cells *in situ* may be calculated to be about

18 pmol/10^7 cells. When thymus was fragmented before homogenization and assay for cyclic AMP, the nucleotide content was increased more than 10-fold (10.445 ± 1.929 nmol/lobe, $n = 7$) corresponding to a cyclic AMP content of about 200 pmol/10^7 cells.

This calculated value agrees well with the cyclic AMP content actually determined in thymocytes prepared by filtration from a thymus mince (220 ± 12 pmol/10^7 cells). During the subsequent steps of thymocyte preparation (centrifugations and resuspensions) the cyclic AMP content remained elevated. After the first centrifugation and resuspension the total cyclic AMP content was 286 ± 19, and after the second centrifugation and resuspension it was 415 ± 32 pmol/10^7 cells. The contribution of extracellular cyclic AMP to the total cyclic AMP was estimated by centrifugation. At this stage of preparation the extracellular cyclic AMP was a minor part of the total cyclic AMP content of the cell suspension. Thus, in the original cell suspension 38 ± 9 pmol/10^7 cells was extracellular, after the first centrifugation and resuspension 28 ± 11 and after the second 25 ± 12 pmol/10^7 cells was extracellular.

After the initial washings, the cells were diluted to a final concentration of 0.2–1.0 × 10^7 cells/ml, and incubation of the cells was started. During incubation of the cells the total cyclic AMP content fell progressively (Fig. 1). The initial fall in cyclic AMP content during incubation was much more pronounced during incubation at 37° than at 4°. Figure 1a shows the pooled data from five experiments. In Fig. 1b is shown one experiment in which a cell suspension prepared at 4° was incubated either at 4° or at 37°, and where one portion was incubated first at 4° for 30 min and thereafter at 37°. This experiment clearly demonstrates the temperature dependence of the cyclic AMP disappearance during incubation.

As shown in Fig. 2 the temperature was an important parameter not only for the rate of decrease during incubation, but also for the absolute level of the cyclic AMP at the start of incubation. The results indicate that a decreased temperature during isolation leads to a decreased cyclic AMP level.

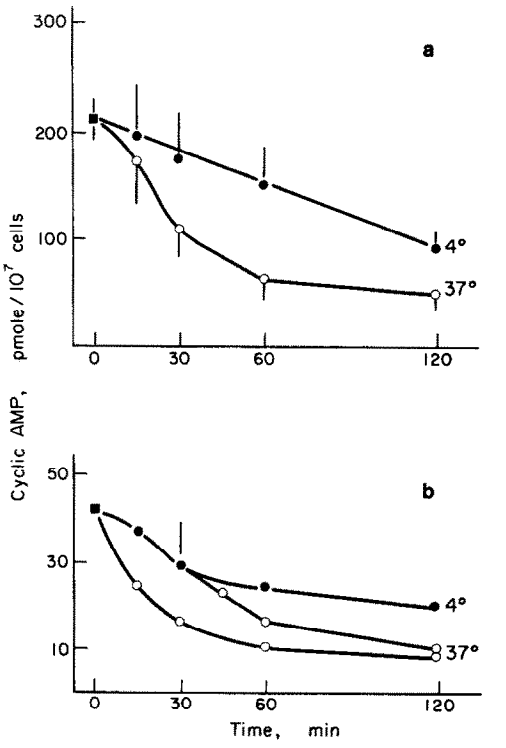

Fig. 1. The effect of temperature on thymocyte cyclic AMP levels. After isolation the cells were incubated either at 37° or at 4°. The upper panel (a) presents results (mean ± S.E.M.) from four separate experiments. The lower panel (b) presents results from two experiments, in both of which some cells were incubated first in the cold, then moved after 30 min to a 37° water bath (at arrow).

During the incubation the extracellular cyclic AMP content remained essentially constant, while the cellular level decreased (Fig. 3). After 60 min of incubation most of the nucleotide was extracellular. The intracellular cyclic AMP content after 60 min of incubation (19 pmol/10^7 cells) was close to the calculated *in vivo* level.

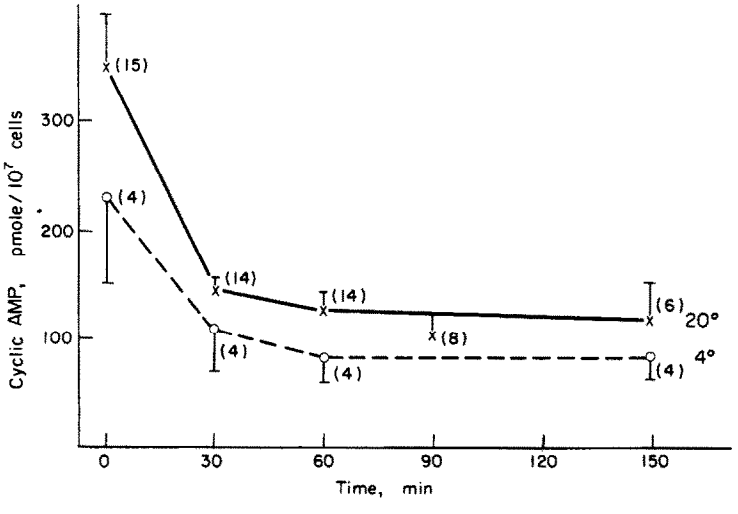

Fig. 2. Cyclic AMP in thymocyte cultures prepared at room temperature (20°) or in the cold room (4°). After preparation the cells were incubated at 37° for the time indicated. Number of experiments at each time point is indicated. Mean ± S.E.M.

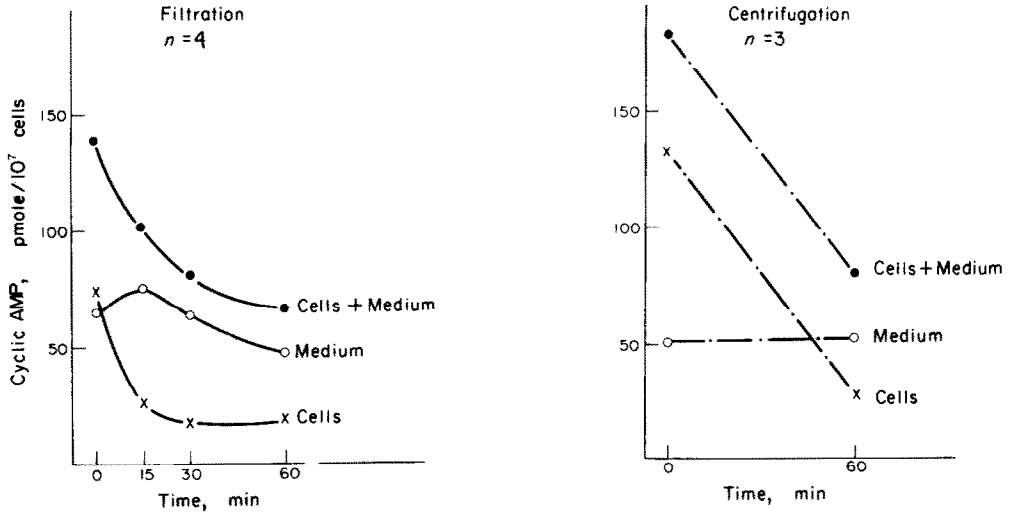

Fig. 3. Intra- and extracellular cyclic AMP in incubated thymocyte suspensions. The content of cyclic AMP in the total suspension, the filtrate after Millipore filtration and in the supernatant after centrifugation was determined at various times after the start of the incubation. Mean of 4 vs 3 separate experiments. The cellular content of cyclic AMP was determined by the difference (or in two experiments by determination on a PCA-extract of the pelleted cells).

Two different approaches were initially used to determine extracellular cyclic AMP (see Methods). Millipore filtrates of cell suspensions were often found to contain higher amounts of cyclic AMP than supernatants obtained by 5 min centrifugation of similar cell suspensions. One explanation for this finding is that extracellular cyclic AMP is broken down during centrifugation. However, there was no significant change in cyclic AMP content of lymphocyte filtrates during centrifugation. Thus the difference in cyclic AMP levels sometimes found between Millipore filtrates and supernatants after centrifugation may instead be caused by cell damage during filtration. The degradation of extracellular cyclic AMP was also studied by adding ^{3}H-cyclic AMP (1 μM final concentration) to a cell suspension (10^7 cells/ml). Less than 10 per cent of the extracellular cyclic AMP was broken down during one hour of incubation at 37° (data not shown). Therefore, it is likely that the fall in cyclic AMP mainly represents intracellular breakdown.

The effects of drugs present during isolation of thymocytes. One possible explanation for the finding that cyclic AMP levels increase during isolation of thymocytes is that some endogenous stimulating substance is released during the procedure. One possible candidate for such a role is a prostaglandin. Therefore the effect of indomethacin was tested. The drug was introduced into the buffers used for preparing the cells at a concentration of 8 μM in the three separate experiments. In these experiments the levels of cyclic AMP was increased rather than decreased. Another possible candidate is adenosine. In order to test the significance of endogenous adenosine several different drugs were used. As shown in Table 1 the presence of adenosine deaminase in the isolation buffers caused a decrease in the cyclic AMP content. Two different inhibitors of adenosine uptake, dipyridamol [33] and dilazep [34] caused a significant increase in the thymocyte cyclic nucleotide content (Table 2). A third inhibitor of adenosine uptake, papaverine [35], caused an even larger increase (Table 3). By contrast the adenosine deaminase inhibitor EHNA, did not cause a significant increase in cyclic AMP content when present in the isolation buffers.

Since theophylline is known to antagonize the effects of adenosine in several systems [15, 36] its effects were tested. The results presented in Fig. 4 demonstrate that theophylline (10^{-3} M) markedly reduces the cyclic AMP content following isolation (from 240 to 70 pmol/10^7 cells). In the control incubates the cyclic AMP level fell during a 90 min incubation. However, in the incubates containing theophylline the levels were unchanged, so that after 60

Table 1. Effect of adenosine deaminase (ADA) on thymocyte cyclic AMP levels

	Cyclic AMP (pmole/10^7 cells)			
	0 min	15 min	30 min	60 min
Exp. 1.				
Control	557	360	194	—
ADA (10 μg/ml)	365	150	116	—
Exp. 2.				
Control	320	—	117	100
ADA (1 μg/ml)	240	—	106	62

The thymus was removed and divided into its two lobes. The lobes were placed in Hank–Dulbecco medium with or without ADA (10 μg/ml or 1 μg/ml dialyzed against the same medium overnight). After preparation and washing of the cells in medium with or without ADA the cells were suspended (final concentration 2×10^6 cells/ml) in RPMI with or without ADA. Samples were taken at 0, 15, 30 and 60 min of incubation at 37°. (Mean of duplicate determinations differing by less than 10%).

Table 2. The effect of phosphodiesterase inhibitors, adenosine uptake inhibitors or EHNA on cyclic AMP levels in the freshly prepared thymocyte suspensions

Addition	Cyclic AMP content	
None	100 ± 2 (16)	
EHNA (10 μM)	110 ± 6 (8)	
Dilazep (30 μM)	176 ± 18 (8)	$P < 0.01$
Dipyridamol (10 μM)	166 ± 17 (8)	$P < 0.01$
IBMX (30 μM)	68 ± 7 (7)	$P < 0.01$
Ro 20-1724 (200 μM)	222 ± 12 (8)	$P < 0.01$
ZK 62.711 (30 μM)	326 ± 26 (8)	$P < 0.01$

The thymocytes were prepared in control (RPMI-) media or in media containing the respective drugs. Incubation for 15–30 min. Results are given in per cent of the corresponding control values (mean ± S.E.M.) which was 238 ± 40 pmole/10^7 cells.

Table 3. The effect of adenosine (10^{-5} M) and theophylline (1 mM) on thymocyte cyclic AMP levels

	Control medium		Papaverine medium	
	pmole/10^7 cells	% of control	pmole/10^7 cells	% of control
Control	50 ± 8		222 ± 13	
Adenosine	86 ± 13	180	394 ± 60	193
Theophylline	32 ± 7	64	143 ± 9	65
Theophylline + adenosine	44 ± 10	88	150 ± 6	70

Thymocyte suspensions were prepared in the presence or in the absence of papaverine. Adenosine and/or theophylline were added and the cells were incubated for 15 to 30 min. Mean ± S.E.M. from 8 separate determinations.

and 90 min of incubation the content was similar in control and theophylline treated cells. A one hundred times lower concentration of theophylline also reduced initial cyclic AMP levels ($P < 0.01$), but in this case the content was further reduced during incubation ($P < 0.05$, Fig. 4). Another methylxanthine, isobutylmethylxanthine (IBMX), similarly reduced the cyclic AMP content of thymocyte suspensions when added to the isolation buffers (Table 2).

Table 4. Effect of EHNA, dilazep, phenylisopropyladenosine (PIA) and adenosine deaminase (ADA) on the cAMP content of incubated thymus cells (pmole/10^7 cells)

Addition	Time after addition of drugs 15 min	60 min
RPMI	124.5	49.4
EHNA, 10^{-5} M	126.7	48.6
10^{-4} M	144.1	73.8
Dilazep $2 \cdot 10^{-4}$ M	153.4	115.1
PIA, 10^{-5} M	159.0	84.5
10^{-4} M	241.8	97.3
ADA, 9 μg/ml	82.5	7.6

The drugs were added after 30 min precinbuation of the thymus cells at 37°. Mean of duplicate determinations at each time point.

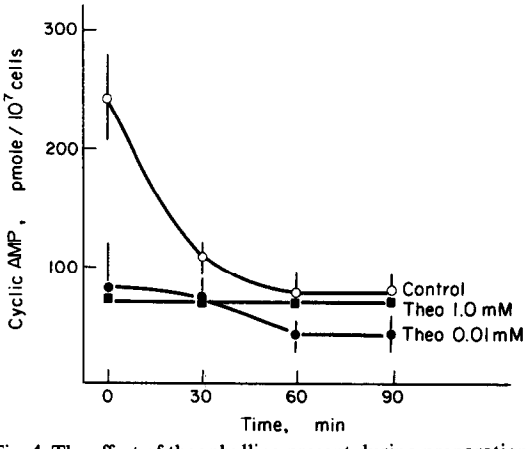

Fig. 4. The effect of theophylline present during preparation of the cells on the cyclic AMP content of thymocytes. The thymus was divided into its lobes; one was placed in control medium. The cells were then prepared as described under Methods using medium with or without theophylline. After dilution to 10^7 cells/ml the cells were placed at 37° and aliquots were taken for cyclic AMP determination. ○ = Control ($n = 6$), ● = theophylline 10^{-5} M ($n = 4$), ■ = theophylline 10^{-3} M ($n = 2$). Mean ± S.E.M., where applicable.

The two methylxanthines, as well as papaverine, are well-known inhibitors of cyclic nucleotide phosphodiesterase. Two non-methylxanthine phospho-

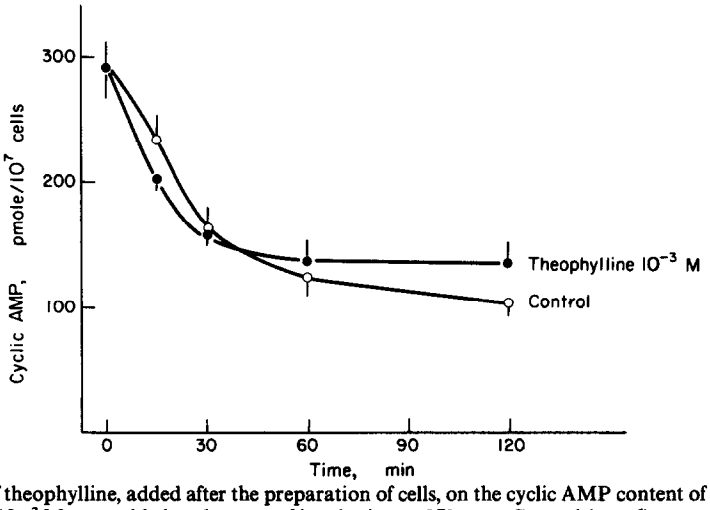

Fig. 5. Effect of theophylline, added after the preparation of cells, on the cyclic AMP content of thymocytes. Theophylline, 10^{-3} M, was added at the start of incubation at 37°. ○ = Control ($n = 6$), ● = theophylline ($n = 4$). Mean ± S.E.M.

diesterase inhibitors, RO 20-1724 and ZK 62.711, are shown in Table 2 to be capable of increasing the cyclic AMP content when added to the isolation solutions.

The effect of drugs added after isolation of thymocytes. Adenosine (Table 3) and its [N^6]phenylisopropyl analogue (PIA) (Table 4) increased cyclic AMP levels when added during the incubation of thymocytes. The uptake inhibitor dilazep as well as EHNA (10^{-4} M) increased cyclic AMP levels (Table 4). Conversely, exogenous ADA decreased the levels.

Theophylline added after the preparation of the suspension caused a slight, but statistically significant, fall in cyclic AMP levels 15 min after addition (222 to 202 pmol/10^7 cells, Fig. 5). At later times the cyclic AMP content of the suspensions was not depressed by theophylline. This suggests that the early fall in cyclic AMP content was not due to enhanced degradation. Furthermore, theophylline depressed cyclic AMP levels also in the presence of papaverine (Table 3). Theophylline antagonized the stimulatory effect of adenosine in the presence and in the absence of papaverine (Table 3). Indomethacin (8 μM) added to the prepared thymocyte suspensions had no significant early effect but tended to decrease the progressive fall in the cyclic AMP content that was observed during incubation (data not shown).

Release of nucleosides during preparation of thymocytes. The above data were compatible with the release of adenosine, or a similar compound, during the preparation of the thymocyte suspension, which stimulated cyclic AMP formation. Attempts were therefore made to study the thymocyte purine metabolism. At 60 min after labelling by an intracardial injection of [^{3}H]adenine (100 μCi) about 215 nCi/g was found in thymus tissue. In the lobe frozen immediately in liquid propane 59% of the total radioactivity was in the form of nucleotides (Table 5). It is seen that upon fragmentation about one third of the tissue nucleotide content is lost. This fall in nucleotide content was parallelled by an increase in nucleosides and bases, and the total radioactivity was unchanged. Most of this non-nucleotide material was found in the supernatant after centrifugation of the cell suspension and consequently may represent material released from the cells. More than 80 per cent of the non-nucleotide material appeared to be hypoxanthine and the remainder was equally distributed as adenine, inosine and adenosine as judged by thin layer chromatography. Using high pressure liquid chromatography the major part of the radioactivity co-chromatographed with hypoxanthine followed by inosine. In addition some 4–10 per cent of the radioactivity co-chromatographed with adenosine (data not shown). Samples taken of the supernatant and cells during further purification of thymocytes revealed a further decrease in cellular radioactivity and a corresponding presence of nucleoside material in the supernatant. For example, in the second wash non-nucleotide material corresponding to 2.3 nCi/g was detected (mean of determinations from two separate animals differing by less than 12 per cent in any of the above figures). Thus, throughout the preparation of the thymocyte suspension there was evidence of further release of purine material from the thymocytes.

DISCUSSION

The present results demonstrate an extensive cyclic AMP formation during the preparation of a thymocyte suspension. The extraction of the thymus and the mincing with scissors produced a ten-fold increase in the thymocyte cyclic AMP content. During subsequent washing a further doubling of the cyclic AMP content was observed. When the cells were incubated at 37° a gradual fall in cyclic AMP was found, so that after 1 hour of incubation essentially basal levels were found. This fall in cyclic AMP appeared to depend both on hydrolysis and on the extrusion of the intracellular cyclic AMP into the medium. The fact that a substantial part of the total cyclic AMP content of the suspensions was extracellular after 60 min of incubation probably explains why the total level was high when calculated on a per cell basis since extracellular cyclic AMP was degraded very slowly. An essentially similar increase in cyclic AMP, followed by a gradual decline during incubation, was observed when lymphocytes were prepared from the spleen and lymph nodes [37].

The content of cyclic nucleotides in guinea pig thymocytes has apparently not been measured previously. Therefore a direct comparison of the present results with previous investigations cannot be made. However, in the rat a level of 173 pmol/10^7 cells [38] and in the mouse levels between 1–2 [39, 40] and 60 pmol/10^7 cells [36] were found. Thus, there

Table 5. Radioactive nucleotides in intact and fragmented thymus following labelling *in situ* with [^{3}H]-adenine 1 hr before sacrifice

		Nucleotides (nCi/g tissue)		Total ^{3}H (nCi/g tissue)		Nucleotides in % of total ^{3}H	
Intact lobe (2)		126		214		59	
Fragmented lobe (2)	Unminced tissue remaining on filter	32	84	47	215	68	39
	Cells	43		66		65	
	Supernatant	8		99		8	

One thymus lobe was frozen directly (intact), the other was minced (fragmented lobe). The latter was then processed as usual for the preparation of thymocyte suspension. Following filtration one part remained on the filter (unminced tissue), the filtrate was separated by centrifugation into cells and supernatant. The total radioactivity and the distribution of radioactivity was determined. The table presents the amount of labelled nucleotides in the different fractions both as nCi/g thymus tissue and in per cent of total radioactivity. Figures within parenthesis gives number of animals.

seem to be large differences due to species and to the technique involved. In particular it is noteworthy that in the mouse, Zenser [36], who found high levels of cyclic AMP, made his determinations on cells + medium, while the other authors [39, 40] who found low levels, made their determinations on the cells only. In the present experiments we found that after about 15 min of incubation at 37° only, about 20 per cent of the total amount of cyclic AMP was actually intracellular.

Since an acute elevation of cyclic AMP during the preparation of the cells may influence the subsequent behaviour of the cells it should be advantageous to reduce this increase as far as possible. We found that the cyclic AMP elevations were reduced by decreasing the temperature, and by inclusion of theophylline or adenoside deaminase in the buffer solutions. It would be of interest to see if such measures alter the immunological competence and proliferative activity of the thymocytes.

Several lines of evidence suggest that the increase in thymocyte cyclic AMP during isolation is due to stimulation of adenylate cyclase by extracellular adenosine (or a related compound). Adenosine is released from several tissues by traumatic stimuli, including hypoxia and ischemia [21–23, 41]. The present results provided evidence that there is release of purine compounds also from thymic tissue. Adenosine, and several adenosine analogs, stimulate cyclic AMP formation in thymocytes [36 and present data], due to activation of adenylate cyclase [36]. The cyclic AMP increase was reduced by decreasing the temperature during preparation of the cells. It is known that adenosine production is reduced by temperature reduction [42, Fredholm and Sollevi, unpublished data]. It was also reduced by the addition of exogenous adenosine deaminase. Two different methylxanthines, theophylline and IBMX, reduced the cyclic AMP response. By contrast, several other cyclic nucleotide phosphodiesterase inhibitors such as papaverine, RO 20-1724 and ZK 62.711 enhanced the cyclic AMP response. Methylxanthines are known to be competitive antagonists of adenosine effects in several different tissues [15, 43–46], including thymocytes [36 and present results]. The potent competitive adenosine deaminase inhibitor EHNA caused a slight, but significant increase in the cyclic AMP response. Finally, inhibitors of adenosine uptake were found to enhance the cyclic AMP response. These results, when considered together, provided support for the opinion that endogeneous adenosine may be released from lymphocytes and that this adenosine causes a transient increase in the cellular cyclic AMP content.

As discussed in the introduction, considerable evidence links an increased adenosine level to the impaired lymphocyte function found in severe combined immunodeficiency. It has been proposed that the effect of adenosine is mediated by an increased cyclic AMP level [5, 36, 47]. Although the exact role of cyclic AMP in lymphocyte function is unclear it has been found that elevated levels of cyclic AMP leads to an impairment of several aspects of thymocyte function [16–20, 47, 48]. Therefore, the present demonstration that released endogenous adenosine could alter thymocyte AMP content may be relevant for our understanding of the pathology of severe combined immunodeficiency.

Acknowledgements—These studies were supported by the Swedish Medical Research Council (proj. no. 2553), Alex and Eva Wallströms stiftelse, Magnus Bergvalls stiftelse and by Karolinska Institutet. We thank Miss Ann-Sofie Larsson, Mrs Louise Vernet and Miss Margareta Widing for skilful technical assistance.

REFERENCES

1. E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet* **2**, 1067 (1972).
2. B. B. Knudsen and J. Dissing, *Clinical Genetics* **4**, 344 (1973).
3. R. Parkman, E. W. Gelfand, F. S. Rosen, A. Sanderson and R. Hirschhorn, *New Eng. J. Med.* **292**, 714 (1975).
4. H. J. Meuwissen, B. Pollara and R. J. Pickering, *J. Pediatr.* **86**, 169 (1975).
5. M. Van der Weyden and W. N. Kelley, *Life Sci.* **20**, 1645 (1977).
6. E. R. Giblett, A. J. Amman, D. W. Wara, R. Sandman and L. K. Diamond, *Lancet* **1**, 1010 (1975).
7. S. H. Polmar, E. M. Wetzler, R. C. Stern and R. Hirschhorn, *Lancet* **2**, 743 (1975).
8. F. F. Snyder, J. Mendelsohn and J. E. Seegmiller, in *Purine Metabolism in Man* (Ed. M. M. Muller), p. **441**. Plenum Press, New York (1977).
9. K. O. Raivio, A. L. Schwartz, R. C. Stern and S. H. Polmar, in *Purine metabolism in Man* (Ed. M. M. Muller), p. 456. Plenum Press, New York (1977).
10. J. W. Smith, A. I. Steiner and C. W. Parker, *J. clin. Invest.* **50**, 442 (1971).
11. H. Green and T.-S. Chan, *Science* **182**, 836 (1973).
12. D. A. Carson and J. E. Seegmiller, *J. clin. Invest.* **57**, 274 (1976).
13. T. Hovi, J. R. Smyth, A. C. Allison and S. C. Williams, *Clin. exp. immunol.* **23**, 395 (1976).
14. K. Ischii and H. Green, *J. cell. Sci.* **13**, 429 (1973).
15. A. Sattin and T. W. Rall, *Molec. Pharma.* **6**, 13 (1970).
16. G. Wolberg, T. P. Zimmerman, K. Hiemstra, M. Winston and L.-C. Chu, *Science* **187**, 957 (1975).
17. F. F. Snyder, J. Mendelsohn and J. E. Seegmiller, *J. clin. Invest.* **58**, 654 (1976).
18. B. V. Jegasothy, A. R. Pachner and B. H. Waksman, *Science* **193**, 1260 (1976).
19. R. Hirschhorn, J. Grossman and G. Weisman, *Proc. Soc. exp. Biol. Med.* **133**, 1361 (1970).
20. D. A. Chambers, D. W. Martin Jr. and Y. Weinstein, *Cell* **3**, 375 (1974).
21. I. Pull and H. McIlwain, *Biochem. J.* **126**, 965 (1972).
22. B. B. Fredholm, *Acta physiol. scand.* **96**, 422 (1976).
23. J. Schrader, F. J. Haddy and E. Gerlach, *Pflügers Arch. ges. Physiol.* **369**, 1 (1977).
24. R. M. Goldblum, F. C. Schmalstcig, A. Nelson, T. Monahan and G. Mills, *Clin. Res.* **24**, 68A (1976).
25. T. G. Brady and C. I. O'Donovan, *Comp. Biochem. Physiol.* **14**, 101 (1965).
26. J. G. Hall, *Aust. J. exp. Biol. med. Sci.* **41**, 93 (1963).
27. B. B. Fredholm, G. Sandberg and U. Ernström, *Acta pharmac. tox.* **41**, Suppl. IV. 48 (1977).
28. B. L. Brown, R. P. Ekins and J. D. M. Albano, *Adv. cyclic nucleotide Res.* **2**, 25 (1972).
29. H. Schimizu, C. R. Crevling and J. Daly, *Proc. natn. Acad. Sci. U.S.A.* **65**, 1033 (1970).
30. E. Böhme and G. Schultz, in *Methods of Enzymology* Vol. 38 (Eds J. G. Hardman and B. W. O'Malley), p. 27. Academic Press, New York (1974).
31. C.-H. Nordström, S. Rehncrona, B. K. Siesjö and E. Westerberg, *Acta physiol. scand.* **101**, 63 (1977).
32. U. Ernström and G. Sandberg, *Acta path. microbiol. scand.* **78**, 362 (1970).

33. H. J. Bretschneider, A. Frank, U. Bernard, K. Kochsiek and F. Scheler, *Arzneimittel-Forsch.* **9**, 49 (1959).
34. J. Pohl and N. Brock, *Arzneimittel-Forsch.* **24**, 1901 (1974).
35. F. W. von Koss, G. Beisenherz and R. Maerkisch, *Arzneimittel-Forsch.* **12**, 1130 (1962).
36. T. V. Zenzer, *Biochim. biophys. Acta* **404**, 202 (1975).
37. G. Sandberg, U. Ernström, K. Nordlind and B. B. Fredholm, *Int. Archs Allergy appl. Immun.* **56**, 449 (1978).
38. C. Spach, U. Srivastava and A. Aschkenasy, *J. Physiol., Paris* **70**, 77 (1975).
39. A. Sevmour, A. Patt and N. Trainin, *J. Immun.* **117**, 2143 (1976).
40. M.-A. Bach, *J. clin. Invest.* **55**, 1074 (1975).
41. B. B. Fredholm and P. Hedqvist, *Br. J. Pharmac.* In press (1978).
42. P. Stevens, R. L. Robinson, K. VanDyke and R. Stitzel, *J. Pharm. Pharmac.* **27**, 864 (1975).
43. A. J. Blume, C. Dalton and H. Sheppard, *Proc. natn. Acad. Sci. U.S.A.* **70**, 3099 (1973).
44. B. B. Fredholm, *Med. Biol.* **55**, 262 (1977).
45. R. Ebert and U. Schwabe, *Naunyn-Schmiedeberg's Arch. Pharmak.* **278**, 247 (1973).
46. B. B. Fredholm, *Acta physiol. scand.* **110**, 191 (1978).
47. T. B. Strom, A. P. Lundin III and C. B. Carpenter, in *Progr. clin. Immunol. Vol. 3* (Ed R. S. Schwartz) 115 (1977).
48. C. W. Parker, T. J. Sullivan and H. J. Wedner, *Adv. cyclic nucleotide Res.* **4**, 1 (1974).