THE METABOLISM OF EXOGENOUS CYCLIC AMP AT LOW CONCENTRATIONS BY THYMIC LYMPHOCYTES*

J. P. MACMANUS, J. F. WHITFIELD and B. BRACELAND

Division of Biology, National Research Council of Canada, Ottawa 7, Canada

Received December 22, 1970

SUMMARY. When isolated thymic lymphocytes were incubated with known mitotically stimulating concentrations (1.6×10^{-7} M and 1.6×10^{-6} M) of a mixture of cyclic AMP labeled with ^{32}phosphorus and tritium, 0.5% of the phosphorus label and 3 to 6% of the tritium label entered the cells. None of the radioactivity from either isotope appearing in the cells was present as cyclic AMP. This difference between the entry of the two isotopes was explained by the observation that the cyclic AMP in the medium surrounding the cells was metabolized, and it was breakdown products of the cyclic nucleotide which entered the cells. It is concluded that cyclic AMP does not enter cells as such, and may exert its action on mitosis at the cell surface.

Low concentrations (5×10^{-9} M to 10^{-6} M) of exogenous cyclic AMP stimulate the initiation of DNA synthesis and thereby increase the proliferation of thymic lymphocytes (thymocytes) maintained in vitro (1-3). Exogenous cyclic AMP also stimulates the proliferation of thymocytes and bone marrow cells in vivo (4). Before the mechanism whereby the cyclic nucleotide stimulates cell proliferation can be studied, it is necessary to know how exogenous cyclic AMP is metabolized by the cells. To this end the present study was undertaken.

METHODS. Thymic lymphocytes were isolated by methods already described (5). The cells were suspended in a simple glucose-salts medium (Fig. 1, ref. 5), washed once, and resuspended in fresh medium. Samples (10 ml) of this cell suspension were placed into test tubes (16 x 150 mm) which were then

*Issued as NRCC No.

rotated at 40 rpm about their long axes at 37°C. After a preliminary 30 min incubation of the cells, the radioactive cyclic AMP was added.

4.0 μl of ^{32}P-cyclic AMP (3.5 Ci/mmole; International Chemical Nuclear Corp., Irvine, Calif.) and 5.0 μl of 8-^{3}H-cyclic AMP (3.0 Ci/mmole; Amersham Searle, Chicago, Ill.) were added to give a final concentration of 1.6×10^{-7} M, composed of 0.8×10^{-7} M of each isotopically labeled cyclic nucleotide. Ten times these volumes were added when 1.6×10^{-6} M cyclic AMP was required. The cell suspensions were shaken, and 2 ml samples taken immediately and then at various times. The sample taken at zero time allowed a measurement of any radioactivity remaining trapped in intercellular fluid after washing. This 'blank' value was subtracted from all cellular radioactivity estimations in subsequent samples.

The samples were placed in ice-cold tubes, centrifuged at 1000 x g for 2 min and the medium decanted for analysis. The pellet was washed twice in 2 ml of ice-cold medium. The resultant cell pellet was sonicated in 1 ml of 7% perchloric acid. The supernatant was then neutralized with 1 N KOH, the volume adjusted to 2 ml, and the precipitate of perchlorate allowed to form in the cold for 30 min. After centrifugation the supernatant was either sampled for measurement of total radioactivity, or freeze dried. After freeze drying, the residue was dissolved in 400 μl of water and chromatographed.

Samples of the medium and the cell extract were chromatographed on silica gel using the method of Dighe et al. (6). After separation, the spots corresponding to ATP + inorganic phosphate (Pi), ADP, AMP, cyclic AMP, adenosine, and adenine were removed, eluted with 1 ml water, and the slurry suspended in scintillation fluid with 4% Cab-O-Sil (7). Elution of all samples except ATP + Pi and ADP was complete. Recovery of these nucleotides was

assessed by the use of internal standards. Radioactivity was measured on a Nuclear-Chicago Mk I scintillation counter.

To differentiate between ATP and Pi, which were not separated by thin-layer chromatography, the differential extraction of Pi as phosphomolybdate into isobutanol was used (8).

RESULTS. When thymocytes were incubated in the presence of either 1.6×10^{-7} M or 1.6×10^{-6} M of a mixture of ^{32}P- and ^{3}H-labeled cyclic AMP, a steady increase in the total intracellular radioactivity due to each isotope was observed (Fig. 1). No difference in the total radioactivity in the medium could be measured (Fig. 2). However after 30 min it can be seen that whereas only 0.5% of the ^{32}P present in the medium entered the cells, between 3 and 6% of the ^{3}H did so. Therefore a greater proportion of the ^{3}H label in the adenine ring entered the cells than could be accounted for on the basis of the entry of ^{32}P-cyclic AMP.

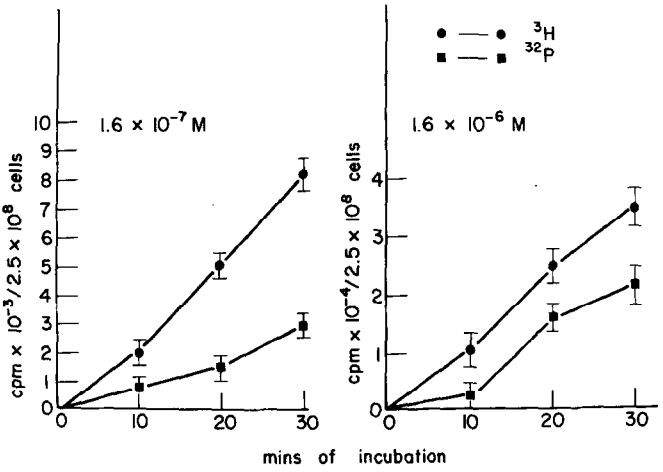

Fig. 1. The accumulation of total radioactivity in rat thymocytes exposed to 1.6×10^{-7} M and 1.6×10^{-6} M cyclic AMP. The cells were incubated in a medium composed of 5.5 mM glucose, 5.0 mM KCl, 120 mM NaCl, 1.0 mM $MgSO_4$, 0.6 mM $CaCl_2$, 5.0 mM Na_2HPO_4 and 5.0 mM tris(hydroxymethyl)-aminomethane buffer pH 7.2. The cell concentration was always 2.5×10^8 cells/ml of medium. ■——■, radioactivity due to ^{32}P; ●——●, radioactivity due to ^{3}H. Results are the mean ± SEM of 12 determinations.

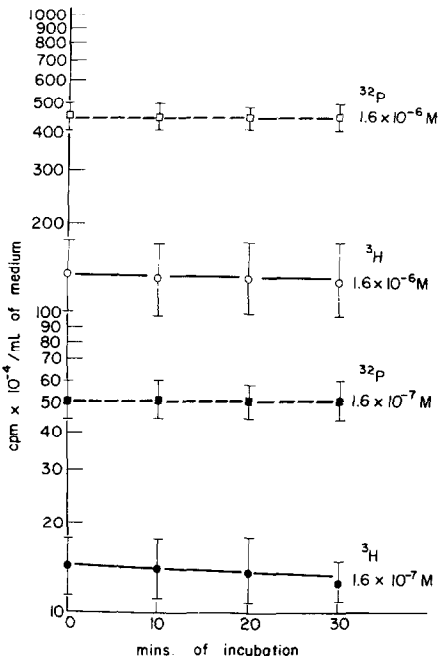

Fig. 2. The total radioactivity present in the medium of cell suspensions after various times of incubation. ■—■ or □—□, radioactivity due to ^{32}P; ●—● or O—O, radioactivity due to ^{3}H. Results are the mean ± SEM of 12 determinations.

However when the cell extract at 30 min was chromatographed, it was observed that none of the radioactivity due to ^{32}P or ^{3}H present in the cells appeared as cyclic AMP, adenosine or adenine after exposure to either initial cyclic AMP concentration (Fig. 3). There was little or no label in AMP, and ^{3}H appeared as both ATP and ADP. ^{32}P was present in the ATP + Pi spot, and also in the ADP spot (Fig. 3). Extraction of ^{32}P into isobutanol indicated the presence of ^{32}Pi in the ATP + Pi spot. Since Pi did not enter the ADP spot in the chromatographic system, the ^{32}P in the ADP spot was concluded to have been in the form of ADP-^{32}P. Thus exogenous cyclic AMP did not increase the intracellular pool of the cyclic nucleotide. The cyclic AMP was either hydrolyzed as soon as it entered, or its label entered the cell as

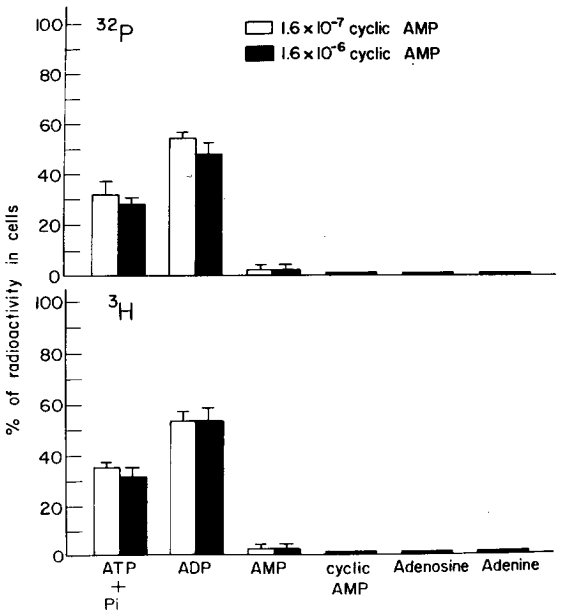

Fig. 3. The distribution of radioactivity in cells after 30 min of incubation in medium containing labeled cyclic AMP. Results are the mean ± SEM of 8 determinations.

---

a metabolite of cyclic AMP. The latter seemed the more likely because of the differential entry of ^{32}P and ^{3}H (Figs. 1 and 2).

When samples of the medium at various times were analyzed, it was found that although 100% of the radioactivity was initially present as cyclic AMP, by 30 min only 65% appeared as such (Fig. 4). A study of the distribution of radioactivity in the medium at 30 min showed that cyclic AMP was metabolized to AMP, since some radioactivity due ^{32}P and ^{3}H appeared in the AMP spot (Fig. 5). ^{32}P also appeared in the ATP + Pi spot (Fig. 5). Extraction of the latter spot into isobutanol indicated that this was Pi. The AMP must therefore have been dephosphorylated. The adenosine or further breakdown products so formed must have rapidly entered the cells, since they did not accumulate in the medium (Fig. 5). This rapid entry of breakdown

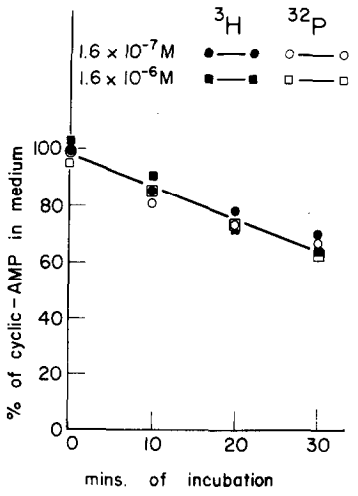

Fig. 4. The proportion of radioactivity present as cyclic AMP in the medium of thymocyte suspensions after various times of incubation. The levels are the means of 8 determinations.

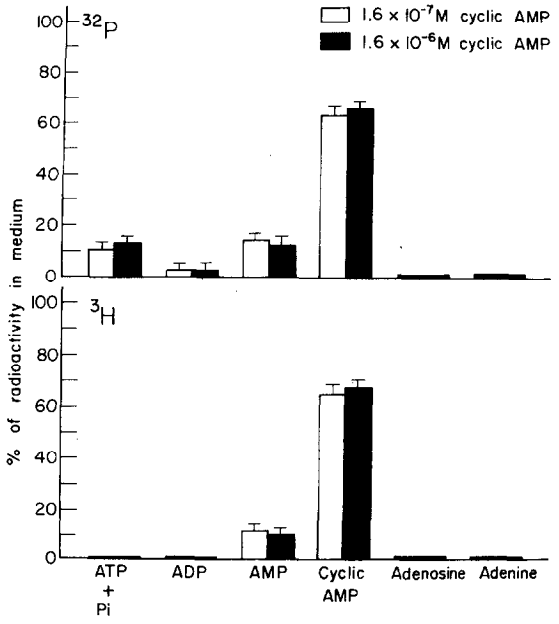

Fig. 5. The distribution of radioactivity in the medium of thymocyte suspensions after 30 min of incubation. Results are the mean ± SEM of 8 determinations.

products could therefore have led to the disproportionate amount of ^3H accumulating in the cells (Fig. 1). However the nature of the breakdown

product could not be identified since it did not accumulate in the cells either, but was rapidly incorporated into ADP and ATP (Fig. 3).

DISCUSSION. Extracellular concentrations of cyclic AMP which stimulate thymocyte proliferation (1-3) did not increase the intracellular pool of cyclic AMP (Fig. 3). The radioactivity from cyclic AMP which appeared in the cells was in the form of breakdown products possibly generated extracellularly (Figs. 4 and 5). The entry of these breakdown products must be irrelevant to the cyclic nucleotide's stimulation of cell proliferation since 5'-AMP, the principal breakdown product of cyclic AMP, is mitogenically ineffective (1, 2, 4). These observations indicate that the cyclic nucleotide need not enter the cells to initiate DNA synthesis, but it may act at the cell surface. Indeed such a hypothesis would explain why such low concentrations of cyclic AMP are so effective both in vitro and in vivo (1, 2, 4). It would also explain the equal molar effectiveness of cyclic AMP and its more permeable dibutyryl derivative in stimulating cell proliferation in vitro and in vivo (2, 4). Such an action at the cell membrane would also explain the complete inability of cyclic AMP to promote DNA synthesis in isolated thymocyte nuclei (9).

REFERENCES.

1. MacManus, J.P., and Whitfield, J.F., Proc. Soc. Exptl. Biol. Med., 132, 409 (1969).
2. MacManus, J.P., and Whitfield, J.F., Exptl. Cell Res., 58, 188 (1969).
3. Whitfield, J.F., MacManus, J.P., and Gillan, D.J., J. Cell. Physiol., 76, 65 (1970).
4. Rixon, R.H., Whitfield, J.F., and MacManus, J.P., Exptl. Cell Res., 63, 110 (1970).
5. Whitfield, J.F., Brohée, H., and Youdale, T., Exptl. Cell Res., 36, 341 (1964).
6. Dighe, P.K., Pahuja, D.N., and Shah, D.H., J. Chromatog., 40, 449 (1969).
7. Snyder, F., and Stephens, N., Anal. Biochem., 4, 128 (1962).
8. Martin, J.B., and Doty, D.M., Anal. Chem., 21, 965 (1949).
9. Burgoyne, L.A., Waqar, M.A., and Atkinson, M.R., Biochem. Biophys. Res. Commun., 39, 918 (1970).