The table shows the results obtained on nine myoglobins in comparison with the data available in the literature and obtained by using the same computation method. Consistent similarities exist among the different sets of data except for the data taken from McLendon[3] which are considerably lower than those presented in this paper and those available in the literature. The small discrepancy between our $\Delta G_{D^2O}^{H_2O}$ values and those taken from Puett[11,12] is due to the numerical value of k (0.6 in our computation, 1.2 in Puett's computation).

The figure shows a plot of the logarithm of the reciprocal of the metabolic rate vs the free energy of unfolding of the myoglobins examined. This kind of plot has already been used to correlate metabolic rates and susceptibilities to unfold of different protein classes[3,4]. However we tried to plot the data in several different ways in order to have a correlation but the results were meaningless. Basal metabolism data were taken from the classic compilation of Brody[13]. No linear correlation appears to exist between the two sets of data; in fact, the least square analysis of the data gave a very poor correlation factor[14], i.e. $r^2 = 0.14$. Therefore, it appears evident that the linear correlation found by McLendon is accidentally limited to the four myoglobins examined by the same author, i.e. dog, horse, cow and whale myoglobins. Widening the number of animal species leads to the conclusion that no simple correlation between the free energy of unfolding and the metabolic rate exists. It is worth taking into consideration that $\Delta G_{D^2O}^{H_2O}$ is a temperature-dependent function; therefore one might expect that the correlation shown in the figure would be improved if $\Delta G_{D^2O}^{H_2O}$ determinations were made at physiological temperatures rather than 25 °C. Among the proteins considered in this paper, seven were from warm-blooded animals with similar physiological temperatures, and the other three from poikilotherm species. Privalov and Khechinashvili[15] reported recently that the free energy of unfolding of myoglobins is fairly constant between 20 and 40 °C and drops at temperatures lower than 20 °C as well as higher than 40 °C. In this respect, the correlation betwen the two sets of data reported in the figure would be further weakened. In fact, while the $\Delta G\Delta G_{D^2O}^{H_2O}$ of myoglobins of warm-blooded animal species is expected to be quite similar to that obtained at 25 °C, a fall in temperature from 25 to 5 °C results in a decrease of $\Delta G_{D^2O}^{H_2O}$ for poikilotherm myoglobins as shown by Puett in the case of turtle myoglobin (table). Moreover, it must be pointed out that these results extend similar observations made previously on homologous cytochrome c's[15].

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5′-Methyl-cytosine in the macronuclear DNA of *Blepharisma japonicum*

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Summary. Brief report on the presence of 5′-methyl-cytosine as a minor base (0.56%) in the macronuclear DNA of the ciliate protozoan *Blepharisma japonicum*. The evidence comes from electrophoresis of macronuclear DNA digested by appropriate restriction endonucleases and high-performance liquid chromatography.
Key words. Protozoa, ciliate; ciliates; *Blepharisma japonicum;* DNA, macronuclear; 5′-methylcytosine.

Methylated adenine (MeAde) and methylated cytosine (MeCyt) in nuclear DNA occur widely in a great variety of organisms from prokaryotes to plants and animals[1].

This base methylation of nuclear DNA has mainly been considered for its relevance in DNA information transfer and much less from the evolutionary point of view. There is good evidence for a relationship of base methylation with the restriction/modification systems in prokaryotes[1] and with other phenomena of DNA function as well[2]. Restriction/modification systems have not been clearly demonstrated in eukaryotes[3], but other functions have been postulated for base methylation, such as the involvement of cytosine methylation in spontaneous mutagenesis, in the determination of the higher order of chromosome structure, and particularly in the control of gene transcription during differentiation[4].

From the evolutionary point of view it is interesting to note that methylation of both adenine and cytosine may occur in prokaryotes[5], though in some species only one base becomes methylated. In contrast, MeCyt is the only methylated base yet found in multicellular eukaryotes. The only exceptions we know of are cultivated cells of *Aedes albopictus,* and salmon sperm, in which a small amount of MeAde has been found[6,7]. The situation in unicellular eukaryotes (protists) is more variable than in multicellular eukaryotes. In some phytoflagellates both MeAde and MeCyt were found, and in dinoflagellates 5-hydroxymethyl-uracil is predominantly present together with

MeCyt in some species[8,9]. Among ciliates, the most animal-like protists, many species of *Paramecium*[10], of *Tetrahymena*[11,12] and two species of the hypotrichous genera *Stylonichia* and *Oxytricha*[13,14] contain MeAde and no MeCyt in their nuclear DNA.

The small number of species yet analyzed among protists does not allow any definite conclusion to be drawn, but the finding of both MeAde and MeCyt or the prevalence of the latter in autotrophic, unicellular eukaryotes contrasts with the presence of only MeAde in ciliates.

During an investigation of the characteristics of the macronuclear DNA (MaDNA) of the ciliate *Blepharisma japonicum*[15] methylated bases were searched for, and here we report on the presence of MeCyt in the MaDNA of this species.

Materials and methods. Culture. A mating type I albino clone A-5/3 of *Blepharisma japonicum* was used. The protozoa were grown in bacterized lettuce juice-phosphate buffer, pH 6.8, according to Miyake et al.[16]. Cells were starved, washed many times and suspended in sterile SMB[17] for 24 h before using.

Purification of macronuclei and DNA extraction. Macronuclei were purified as previously described by Salvini et al.[15]. The packed cells were incubated for 5 min in an equal volume of a buffer containing 20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 3 mM $CaCl_2$ (solution A); to the suspension an equal volume of 0.9% Triton X-100, 1 M citric acid, pH 1.5, was added and the cells were gently homogenized with the aid of a Pasteur pipette. The macronuclei were recovered by centrifugation, resuspended in the solution A and centrifuged at 50 × g several times. Then the macronuclei were suspended in solution A to which an equal volume of a solution containing 2 M saccharose, 20% glycerol, 3 mM $CaCl_2$ and 100 mg/ml spermine (solution B) was added.

One volume of this suspension was layered over 20 volumes of the solution B and the macronuclei formed a pellet after centrifugation for 5 min at 3000 × g.

DNA extraction was carried out according to the technique described by Swanton et al.[18].

Restriction endonuclease digestion and electrophoresis of macronuclear DNA. MaDNA from two independent extractions was digested with restriction enzymes Hpa II and Msp I (Boehringer, Mannheim) according to the suggestions of the supplier for 2–5 h at 37 °C. λ-Phage DNA restricted with the same enzymes was used as an internal or an external marker.

DNA fragments were electrophoresed through 1.0% agarose gels at 30 V overnight, stained with ethidium bromide (1 μg/ml) and photographed with a polaroid MP3 land camera under ultraviolet light.

DNA hydrolysis and HPLC conditions. 5'-Methyl-cytosine determinations were carried out according to Citti et al.[19].

Hydrolysis of DNA samples (10 μg each) was performed in a sealed glass vial with 100 μl of 88% formic acid at 175 °C for 30 min. Hydrolysates were then lyophilized and the residues solubilized in 0.1 N HCl; the solutions were neutralized at pH 5 with 1 N KOH and filtered through an 0.45 μm cellulose filter.

Samples were loaded on a Cg reversed-phase column equilibrated at 1 ml/min (about 85 at pressure) with a mixture of 1% of diluted methanol (70% v/v solution in H_2O) and 99% of 20 mM K-phosphate buffer containing 5 mM sodium hexane sulphonate, pH 5.4. Chromatographic runs were performed isocratically at starting conditions for 12 min, thereafter with a linear gradient up to 20% of eluent in 13 min. The

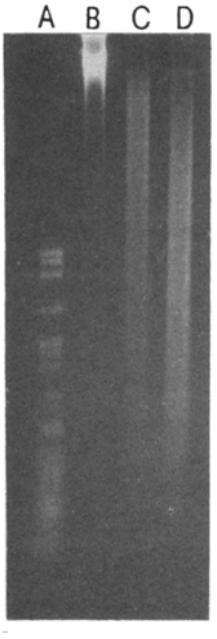

Figure 1. Restriction digests of λ-DNA and of macronuclear DNA (MaDNA): A) λ-DNA digested with Hpa II; B) uncut MaDNA; C) MaDNA digested with Hpa II; D) MaDNA digested with Msp I.

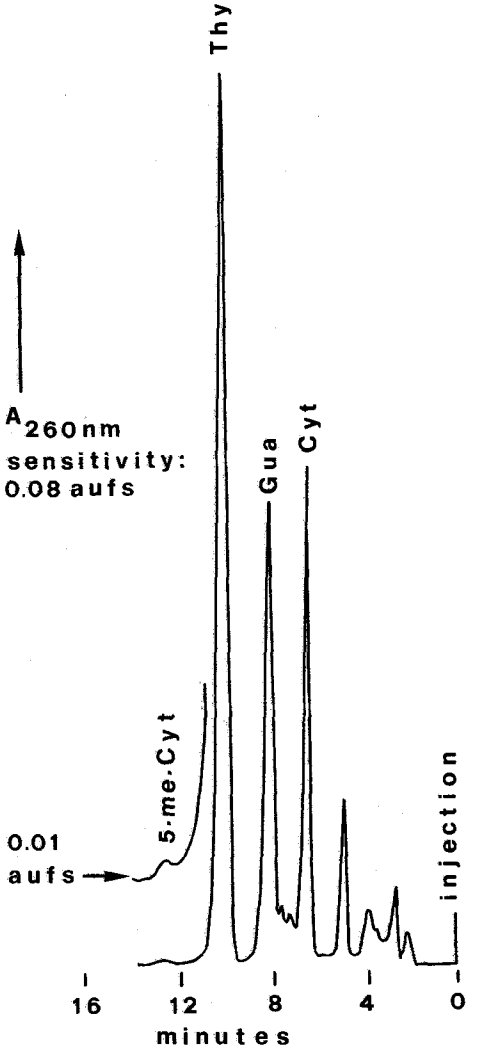

Figure 2. HPLC analysis of macronuclear DNA: chromatographic run is from right to left. The peak indicated as 5'-MeCyt in the upper expanded chromatogram, had the same retention time as the pure standard.

mole percentages of the bases were determined from the area of the peaks in the elution pattern compared with the areas of calibration curves made by using pure bases as standards.

Results and discussion. The agarose gel electrophoresis of the restricted MaDNA is shown in figure 1. The two restriction endonucleases used, Hpa II and Msp I, both recognize the sequence 5'-CCGG but while Hpa II does not cleave when internal cytosine is methylated, Msp I cleaves the sequence regardless of methylation. Msp I digestion of MaDNA produces shorter fragments than Hpa II digestion. The fact that Msp I is more active than Hpa II points to the presence of MeCyt in the *Blepharisma japonicum* genome.

This result is confirmed by HPLC analysis (fig. 2). A clear peak occupying the same position of standard MeCyt is seen after 12 min elution from sample injection. The adenine retention time is 20.2 min not shown in figure (see Citti et al.[19]). The MeCyt content corresponds to about 0.56 mole per cent of the total cytosine; this amount comes from the averaged value of four replicated analyses of two distinct MaDNA extractions. The minor peaks present immediately after injection represent other minor bases not yet well identified and partially hydrolyzed DNA (unpublished results). This fact may cause an underestimate of the amount of MeCyt in MaDNA.

The importance of this short report resides in the demonstration of the presence of MeCyt in the MaDNA of a ciliate protist. The MaDNA from all ciliates examined up to now has only been found to contain MeAde. Our finding contrasts *Blepharisma japonicum* with other ciliates but it aligns this species with the other unicellular autotrophic eukaryotes that make use of cytosine methylation like *Euglena*[20], *Chlamydomonas*[12], and *Exuviella*[9]. In prokaryotes both adenine and cytosine can be methylated, whereas in multicellular organisms MeCyt is the most widespread or the unique modified base. Among ciliates, considered to be the most animal-like of all protists, the methylation of cytosine residues seems to have disappeared in many species, but it persists in *Blepharisma*.

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The effect of mouse erythrocyte rosette forming lymphocytes on lymphokine production in T-cell cultures

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Summary. An enhancement effect of mouse erythrocyte rosette forming (MERF) cells on the production of migration inhibitory factor, chemotactic factor for neutrophils and skin reactive factor in T-lymphocyte cultures stimulated with the purified protein derivative of tuberculin was observed. We consider it likely that the MERF cells, possessing the appropriate cell surface constituents to construct an immunogenic moiety, present antigen on their surfaces to elicit lymphokine production.
Key words. Lymphokines; mouse erythrocyte binding lymphocytes; T-lymphocytes.

Lymphocytes stimulated by a mitogen or a specific antigen produce a number of soluble factors (lymphokines) with various activities. These include macrophage migration inhibitory factor (MIF), chemotactic factor for neutrophils (CFN), skin reactive factor (SRF), etc. These factors play a part in the inflammatory processes of delayed-type hypersensitivity. Both T- and B-cells are able to produce lymphokines[2,3], but the interactions between these two populations of lymphocytes in lymphokine synthesis have been studies less extensively.

Lymphocytes forming rosettes with mouse erythrocytes (MERF cells) have been detected in the peripheral blood. The MERF lymphocytes proved to be B-cells which have surface immunoglobulin and receptors for C3 which do not possess T-cell specific markers[4,5]. Rosette formation with mouse erythrocytes (ME) seems to define a population unresponsive to pokeweed mitogen and to be a marker for resting B-lymphocytes[6]. ME rosette-positive B-cells stimulate poorly in autologous and allogenic mixed lymphocyte reactions[7].

The aim of this investigation was to study the effect of MERF cells on the production of MIF, CFN and SRF by T-lymphocytes stimulated with a purified protein derivative of tuberculin (PPD), and thus to obtain data on the role of this B-cell subpopulation in T- and B-lymphocyte interactions.

Methods. Samples of blood were collected from 31 PPD skin test-positive healthy volunteers. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation, using the method described by Böyum[8].

T-lymphocytes were isolated from a PBMC suspension by a positive selection method. Aliquots of PBMC partially depleted of adherent cells, and sheep red blood cells (SRBC) were mixed at a final ratio of SRBC to PBMC of 20:1, centrifuged for 3 min at 400 g at room temperature, and then incubated