(0.15 mmol) in acetone-$d_6$ (0.5 ml); methanol-$d_4$ (0.2 ml) was added and after 24 h (N–H to N–D) the curves were recorded again. The singlet ($\delta$ 8.6–8.8) of the picrate anion was taken as proton-counting reference; the number of N–H resulted directly from the integration curves or from the difference with those obtained after addition of methanol-$d_4$. The number found for N-H was usually within 0 to $-10\%$ of the correct one.

The spectra do not show separated signals for E–Z isomers[14]. The N–H signals are generally characterized by: $\delta$-values in the range 6.7–8.2; half-band width ($W_{1/2}$) 5–18 Hz; they disappear with methanol-$d_4$; $\delta$-values are not changed or are slightly decreased (less than 0.1 ppm) by methanol. An aryl substituent produces: a strong downfield shift of the Ar–N–*H* signal; a small effect on the protons of the other N-atoms; the N–H resonances disappear ('averaged' with MeO–*H*) or are strongly broadened by methanol.

The aromatic-H signals are distinguished because they are not removed by methanol-$d_4$. The N–H signals of amines (as picrate salts) are usually very broad ($W_{1/2}$ > 40 Hz) and disappear by methanol. The O–H ($W_{1/2}$ ca. 2 Hz) or N–H ($W_{1/2}$ > 40 Hz) signals of phenols and carboxamides are shifted downfield (0.2–0.3 ppm) by methanol.

After the presence of the guanidinium group has been ascertained, its substitution pattern results in many examples from the N–H signals in the spectra measured in plain acetone. Thus, an example ($R^1 = R^3$ = cyclohexyl; $R^2 = R^4 = R^5 = H$) exhibits 4 N–H distributed in 2 signals ($\delta$ 7.06 and 7.26; area ratio 1:3) partially superposed; these data indicate non-equivalent N–H groups, and therefore the compound is a $N_1,N_2$-disubstituted guanidinium. The observed area ratio is due to superposition of the lower-field component of the splitted N–H signal (doublet; two H–N–C–H) with the $NH_2$ singlet. The splitting is confirmed by decoupling with ferric chloride.

The scope is widened, including the results in acetone-$d_6$, which allows the observation of the N–C–H signals at $\delta$ 3.0–4.3; an adjacent aryl group (N–C*H*–Ar) produces a downfield shift ($\delta$ 4.5–4.9). The method now takes into account: number of guanidinium N–H; occurrence of a singlet or more N–H signals arising from non-equivalence or spin-spin coupling with N–C–H; number of N–C–H multiplets simplified by methanol-$d_4$.

One example is given in detail ($R^1 = R^2$ = ethyl; $R^3$ = methyl; $R^4$ = H; $R^5$ = benzyl) which gives a sharp signal at $\delta$ 7.33 that in its base is partially superposed with a broad signal (total area 7 H). After addition of methanol-$d_4$ it remains a 5H-singlet (aromatic-H), indicating that the compound is a tetra-substituted guanidinium. Besides, methanol-$d_4$ simplifies two doublets to give the corresponding singlets at $\delta$ 3.00 and 4.55; this implies that two of the substituents are located on N-atoms bearing a proton responsible of the N–C–H splittings. Then, the compound is a $N_1,N_1,N_2,N_3$-tetra-substituted guanidinium.

Some of the compounds were also measured using plain and hexadeuterated dimethylsulphoxide as solvents; the results were generally similar to those quoted above. These solvents are mainly useful for low soluble picrates (e.g. $R^1 = R^2$ = methyl; $R^3 = R^4 = R^5 = H$).

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## Double Stranded Ribonuclease Activity in Human Lymphocyte Nuclei

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*Summary.* Ribonuclease activity directed against the synthetic duplex polyrC:polyrI was detected in nuclear extracts from both unstimulated and PHA-stimulated human lymphocytes. In the latter cells, the activity was about twice that of small lymphocytes.

It has been shown that human small lymphocytes synthesize mainly unmethylated rapidly sedimenting RNA molecules which hybridize very efficiently to DNA in vitro and are bound to polyadenylic sequences in a proportion up to 20%[1,2]. It was thus concluded that the major portion of the RNA synthesized in these cells is the heterogeneous nuclear RNA. Since it has been shown by several authors that a significant portion of nuclear RNA of the heterogeneous type in animal cells is in an RNase-resistant form with properties of double-stranded

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Ribonuclease activity directed against single-stranded and double-stranded synthetic polyribonucleotides in nuclei of unstimulated and PHA-stimulated normal human lymphocytes

| Donor No. | Substrate | Small lymphocytes | | PHA-stimulated lymphocytes | |
|---|---|---|---|---|---|
| | | Control incubation (cpm) | Substrate radioactivity hydrolyzed (%) | Control incubation (cpm) | Substrate radioactivity hydrolyzed (%) |
| 1 | $^3$H-polyC-polyI | 6450 | 11 | 6450 | 25 |
| | $^3$H-polyrC | 5100 | 12 | 5100 | 15 |
| 2 | $^3$H-polyrC-polyrI | 4430 | 13 | 4430 | 27 |
| | $^3$H-polyrC | 4500 | 11 | 4500 | 13 |
| 3 | $^3$H-polyrC-polyrI | 5200 | 27 | 5200 | 56 |
| | $^3$H-polyrC | 2800 | 18 | 2800 | 21 |

After dissolution of the nuclei in 1 *M* NaCl buffer, the extract was dialyzed against 0.14 *M* NaCl buffer and incubations performed in buffer of this molarity.

RNA[3–7], it was not unexpected to observe that about 2% of the total labelled cell RNA of small lymphocytes shows double-stranded properties[8].

At variance with that observed in several animal cell types, the turnover rate of heterogeneous nuclear RNA in small lymphocytes appears remarkably low, as suggested by the association of the majority of a labelled precursor with rapidly sedimenting molecules even after several hours of actinomycin chase[1,2]. This conclusion is also supported by the observation that cold uridine chase for several hours does not significantly change the proportion of labelled double-stranded RNA[8,9].

Phytohemagglutinin (PHA) stimulation of small lymphocytes brings about a sharp change in metabolism of nuclear lymphocyte RNA, characterized by a large increase in the rate of synthesis and processing of ribosomal precursor RNA, as well as by increase in turnover rate of the heterogeneous nuclear RNA[1,2]. Very little information is so far available about enzyme systems involved in metabolism of nuclear lymphocyte RNA. The presence of double-stranded RNA segments in heterogeneous nuclear RNA justifies the hypothesis that the cleavage of the large RNA molecules of this RNA class involves the activity of an enzyme with a definite preference for double-stranded RNA, in a manner similar to what has been recently demonstrated in bacterial cells. In these cells, in fact, it was recently observed that ribonuclease III, an enzyme highly specific for double-stranded RNA, has a key role in processing of messenger as well as ribosomal RNA precursors[10–13].

This paper shortly reports the results of a group of experiments carried out in our laboratory to assay in lymphocyte nuclei, before and after PHA stimulation, the activity of a ribonuclease able to digest double-stranded regions of RNA.

*Materials and methods.* Human small lymphocytes were obtained from the peripheral blood of 3 normal donors. Heparinized blood was sedimented by gravity and lymphocyte suspensions, with granulocyte contamination lower than 0.5%, were prepared by filtration of the supernatant through commercial nylon fibres, according to the technique already described[1]. Cultures were set up in Eagle's medium with 20% autologous plasma at the average cell concentration of $2 \times 10^6$ cells per ml. The total lymphocyte yield from each donor (2.6 to $3 \times 10^8$ lymphocytes) was divided into 2 equal parts, one of which was immediately harvested and the nuclei separated. The other part was incubated at 37 °C for 24 h after addition of PHA solution (Wellcome, Reagent Grade) at the concentration of 0.02 ml/ml, and then harvested. Nuclei of high purity were obtained without mechanical shearing by a minor modification of the method of TAKAKUSU et al.[14], using a mixture of detergent solutions containing sodium deoxycholate and Triton WR 1339. The harvested cells were washed twice with 50 ml of sucrose buffer (0.15 *M* sucrose, 0.005 *M* $CaCl_2$, 0.025 *M Tris* · HCl, pH 7.1). The washed cells were resuspended in 15 ml of the same buffer, and an equal volume of detergent solution (0.25% sodium deoxycholate, 0.5% Triton) was added, and the suspension shaken gently by hand for 5 min. The suspension was then diluted with 100 ml of sucrose buffer to prevent further detergent activity, and then centrifuged at 1500 rpm for 10 min, to eliminate the cytoplasmic components in the supernatant. The resulting pellet of nuclei was washed thrice with 20 ml of 0.25 *M* sucrose buffer at 4 °C. Microscopic examination of Giemsa-stained nuclei showed that the nuclear chromatin and nucleolar structure were well preserved, and that only a very few nuclei retained small cytoplasmic tags. The washed pellet was then easily and evenly dissolved in 10 ml of buffer (1 *M* NaCl, 0.02 *M Tris* · HCl, MgAcet. 0.01 *M*, pH 7.6), and 50–200 µl aliquots were separated and used in the enzyme assay. A portion of this solution was dialyzed for 72 h against 0.14 *M* NaCl buffer. The precipitated deoxyribonucleoprotein was then separated by centrifugation and the supernatant again tested for double-stranded ribonuclease activity.

Synthetic substrate for the enzyme assay was prepared from equimolar amounts of polyrI and polyrC containing


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$^3$H-polyrC. All polymers were from Miles. PolyrI was dissolved in 0.02 *M Tris* buffer (pH 7.8) containing 0.1 *M* NaCl and extracted twice with buffer saturated phenol. The aqueous phase was then dialyzed in the same buffer to eliminate minimal traces of phenol. $^3$H-polyrC was diluted with 0.02 *M Tris* buffer and dialyzed to eliminate ethanol from the commercial preparation. While in initial experiments phenol extraction was performed, this was found unnecessary in further trials. The formation of the bihelical homopolymer duplex $^3$H-polyrC:polyrI was evaluated by the hypochromic effect at 233 nm. The temperature of the product was found between 60 °C and 70 °C in 0.1 *M* NaCl. The duplex was also tested for stability to digestion by RNase B (Worthington) for 30 min at 37 °C. For concentrations of the enzyme up to 1 µg/ml, no effect was observed on the amount of acid precipitable counts of the duplex in most preparations of $^3$H-polyrC:polyrI, whereas the single stranded $^3$H-polyrC was made completely acid soluble. The reaction mixture contained an amount of $^3$H-polyrC:polyrI equivalent to about 0.5 nmoles of P and variable amounts of nuclear extracts in a final volume of 1 ml of buffer (1 *M* or 0.14 *M* NaCl, 0.01 *M* MgAcet. and 0.02 *M Tris* · HCl). The reaction was stopped after 30 min by addition of 2 ml of 10% cold TCA. 50 µg of albumin were added as carrier, and the precipitate was filtered through Millipore filters (HAWP25). The filters were dried and counted in a Packard Tricarb scintillation spectrometer. Assays were always performed in duplicate, and average values are presented.

*Results.* All the nuclear extracts assayed in our experiments displayed a significant digesting activity on the double-stranded substrate. Results obtained with reaction mixtures in 0.14 *M* NaCl buffer are shown in the Table. The proportion of substrate radioactivity digested in buffer of this molarity was always greater than that observed in 1 *M* NaCl buffer, even though control experiments showed that a significant amount of activity was still bound to the precipitate formed after dialysis. The rate of hydrolysis of the labelled duplex was irregular and proportionality to the amount of nuclear extract added to the reaction mixture could not be demonstrated. All the results shown in the Table were obtained with 200 µl of extract, corresponding to about $3 \times 10^6$ nuclei. The activity in this amount of nuclei from unstimulated lymphocytes of donor No. 2 brought to hydrolysis of 0.08 nmoles of double-stranded substrate, whereas the activity in the same number of nuclei, after 24 h of PHA stimulation, brought to hydrolysis of 0.17 nmoles of substrate. Single-stranded ribonuclease activity was present in each of the samples where double-stranded ribonuclease activity was detected. However, in all donors examined, double-stranded ribonuclease activity in nuclei of PHA-stimulated lymphocytes was about twice that before stimulation. On the contrary, only a small average increase in single stranded ribonuclease activity was observed in lymphocyte nuclei after PHA stimulation. Furthermore, hydrolysis of $^3$H-polyrC was markedly stimulated by EDTA, which had, on the contrary, a slight inhibitory action on double-stranded ribonuclease activity. These results suggest that single-stranded and double-stranded ribonuclease activities are associated with different nuclear proteins. Experiments are now in progress in the attempt to separate these proteins.

## Dissociation-Constants of Metal-Ion-Complexes with Alkaline Phosphatase from Pig Kidney

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*Summary.* Using metal-ion buffers it was possible to remove $Zn^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ ions of pig kidney alkaline phosphatase reversibly. The dissociation constants obtained are $K_{EMg}$: $4 \cdot 10^{-7}$ *M*, $K_{EMn}$: $4 \cdot 10^{-8}$ *M* and $K_{EZn}$: $8 \cdot 10^{-13}$ *M* (22 °C, pH: 9.6, µ: 0.07).

Alkaline phosphatase (EC 3.1.3.1) is a metal-containing enzyme. It requires $Zn^{2+}$ ions both for preservation of its structure and for its enzymic activity (e.g. ref.[3]). Zinc may be replaced by cobalt, copper, cadmium, nickel or manganese[4-7]. In addition to these metal ions, the alkaline phosphatase from mammalian tissue needs $Mg^{2+}$ ions for activity[8,9]. Other divalent cations, especially $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$[9] and $Ca^{2+}$ have been reported to be capable of replacing $Mg^{2+}$ as activator. Most of these experiments have been performed in the absence of complexing agents. Therefore, due to contamination of the reagents and of the enzyme preparation, there are various metal ions in the assay. Furthermore, the concentration of metal ions were varied in only a few cases.

We determined the dissociation constants of several metal ion complexes with alkaline phosphatase in the presence of a suitable complexing agent under conditions which guarantee that the removal of metal ions is reversible and that an equilibrium is achieved. Furthermore, we solved the problems arising from a system consisting of more than one sort of metal ion, complexing agent and protein by means of a FORTRAN IV program or an approximate formula.

*Experimental.* All chemicals were obtained from E. Merck, Darmstadt, Germany. Pig kidneys were used as source for the alkaline phosphatase.

*Methods.* The preparation of pig kidney alkaline phosphatase and the determination of enzyme activity was described recently[8,10]. The test medium for kinetic mea-

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