DHT was the main metabolite found in all incubations with Δ^4A as the substrate. The addition of HCG mainly enhanced the formation of 5α/βA, but not significantly that of T and DHT. The addition of NADH resulted in the increased formation of DHT.

In spring Δ^4A was strongly converted to Δ^4A-diol (nearly half the radioactivity). The amount of T was significantly reduced (as compared to winter) but the ratio of DHT/T was higher. The addition of NADH increased both the amount DHT (2-fold) and DHT/T ratio. Δ^4A-diol was significantly reduced. Hibernating frogs are known to live in temperatures below 4 °C[11]. We did not test these temperatures. However, DHA metabolism in testicular homogenate of hibernating hedgehogs has been shown to be reduced but had not changed its pattern when temperature is lowered to 4 °C[9].

The formation of C-19 steroids from DHA and androstenedione in testis tissue of *R. temporaria* showed a marked seasonal variation in the present incubations. Distria et al.[3] have shown that the plasma levels of T in males of *R. esculenta* were maximal from December to March and minimal from August to September. T and DHT plasma levels of *R. pipiens* have been shown to be low during summer months and higher already in October[12]. In our experiments, T was produced at a considerable rate by the testes of winter and spring frogs. DHT was formed from Δ^4A in a substantial rate already in winter frogs. This is in line with the findings of Wada et al.[12] with leopard frog and with those of Müller[13,14] with bullfrog, demonstrating DHT as the major testicular product. Its formation was specifically stimulated by bullfrog LH[13,14]. Furthermore, the formation of DHT from T in incubations of testicular tissue obtained from 2 anuran species (*R. temporaria* and *Discoglossus pictus*) has been previously shown[15,16].

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Cytoplasmic contaminants in Triton X-100 washed rat liver nuclei – a possible way of further purification[1]

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Summary. The presence of cytoplasmic contaminants is demonstrated in preparations of rat liver nuclei, even after washing with Triton X-100. They can be removed by incubation at 37 °C in a medium of low-salt concentration.

In previous experiments[2], when Triton-treated nuclei were incubated with [γ-^{32}P]ATP in low-salt medium[3] in order to phosphorylate their proteins in vitro, components amounting to 10% of the total protein content of the nuclear preparations were solubilized. The extract, called fraction 2, was found to contain extensive cytoplasmic contamination. The application of incubation in low-salt medium is suggested as an additional step in the purification of rat liver nuclei.

Material and methods. Wistar rats of both sexes were given 4 mCi/200 g ^{32}P orthophosphate i.p. 1 h before killing. Nuclei were purified by the method of Blobel and Potter[4], washed in 0.25 M sucrose-3.3 mM $CaCl_2$ (SC), and treated with 1% v/v Triton X-100 dissolved in SC[5]. The low-salt medium (used originally for labelling in vitro[3]) contained 30 mM Tris-HCl, pH 7.5; 0.25 M sucrose; 25 mM NaCl; 10 mM Na_2SO_3; 5 mM Mg acetate and 0.5 mM ATP. The incubation time was 10 min at 37 °C. Saline extract from the incubated nuclei was also separated[5] with 0.14 M $NaCl^-$ 20 mM Tris-HCl, pH 7.5. The different fractions obtained were analyzed as detailed in the figures and the table.

Results and discussion. Figure 1 shows that the electrophoretic profile of fraction 2 differs considerably from that of the 0.14 M NaCl extract. Isopycnic density gradient centrifugation on metrizamide (figure 2) revealed light components in fraction 2. The nucleic acid contents of the pooled fractions, however, apparently cannot be responsible for their low buoyant density. In fact, quantitative analysis of fraction 2 shows a 30% lipid content. This explains the presence of the light components and is also an

Glucose-6-phosphatase activity of different cellular protein fractions obtained during the purification of rat liver nuclei

Fraction	µg P liberated/mg protein
Cytoplasmic components	
12,000 rpm pellet	98.46
40,000 rpm pellet	181.40
40,000 rpm supernatant	17.10
Nuclear components	
0.25 M sucrose, 3.3 mM $CaCl_2$ wash	27.33
0.25 M sucrose, 3.3 mM $CaCl_2$, 1% w/v Triton X-100 wash	22.30
Whole nuclei	5.51
Fraction 2 (supernatant of nuclei after incubation in the medium given in figure 1, B)	32.40
Nuclei after the extraction of F2	2.60
Nuclei, after the extraction of F2, extracted in saline-Tris (figure 1, C)	2.24
Whole nuclei extracted in saline-Tris	2.13

Liver homogenate was first subjected to low-speed centrifugation and cytoplasmic fractions were obtained from the supernatant. G-6-P-ase activity was measured by determining the liberated orthophosphate[11].

obvious indication of cytoplasmic contaminants. When glucose-6-phosphatase (G-6-P-ase) activity was measured from different fractions obtained throughout the purification procedure of the nuclei (table), high activity was found in fraction 2, showing its contamination with particulate cytoplasmic elements. The G-6-P-ase activity of the SC-Triton wash fell well below that of the SC wash and fraction 2. This suggests that washing in SC-Triton is not an efficient way of removing the cytoplasmic contaminants that are extracted later in fraction 2, and the latter may not be considered as a remaining part of the SC-Triton washing. The different electrophoretic profiles (figure 1, A and B) considering either the Coomassie brilliant blue staining or the ^{32}P labelling pattern, lend further support to the observation that the SC-Triton wash and the fraction 2 are not related fractions. These data indicate a limitation of the Triton washing method during the isolation of rat liver nuclei, by revealing the presence of cytoplasmic contaminants that cannot be removed efficiently by the detergent treatment. The presence of this contamination in the nuclear preparations cannot be attributed to some inadequacy in the isolation procedure. Electron microscopic examination revealed that the outer nuclear membrane was

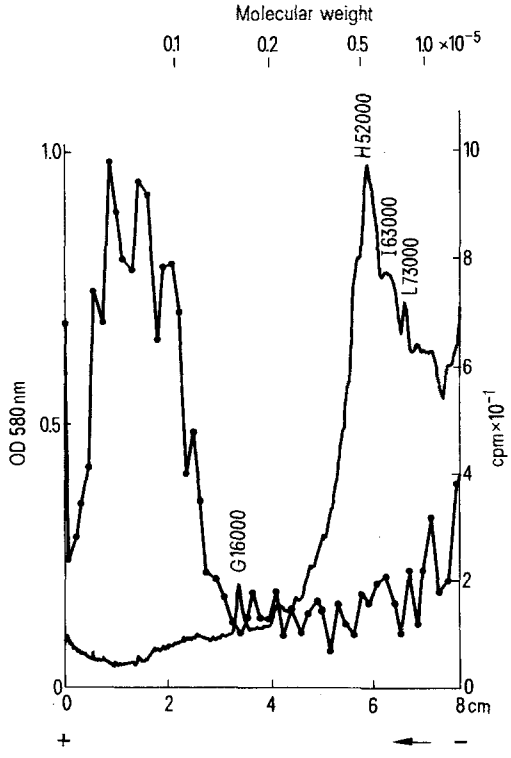

Fig. 1a

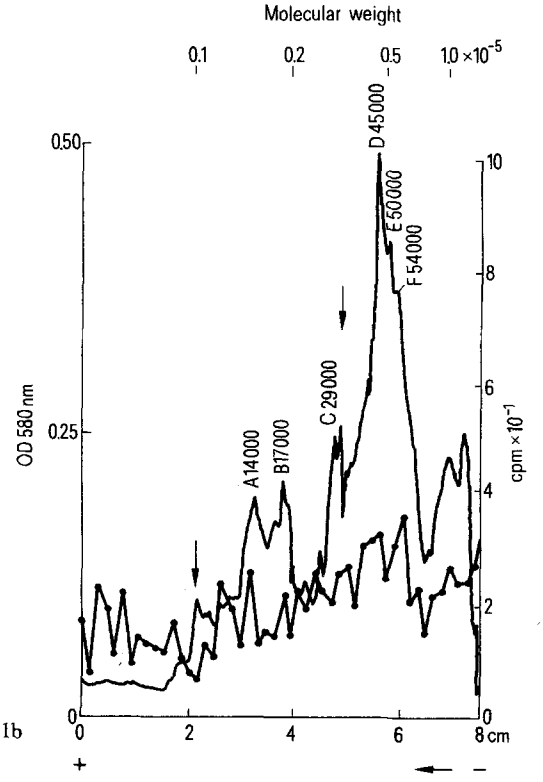

Fig. 1b

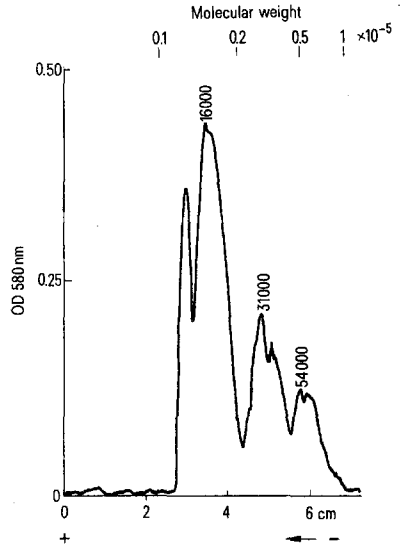

Fig. 1c

Fig. 1. SDS polyacrylamide gel electrophoresis[5,6] of fraction 2 and other soluble fractions of rat liver nuclei. *A* Supernatant of the SC-Triton washing; *B* fraction 2 (supernatant obtained by incubating the nuclei in low-salt medium); *C* 0.14 M NaCl extract. Nucleic acid-P was removed from the gels by hydrolysis[7]. ——: OD at 580 nm; ●——●: radioactivity.

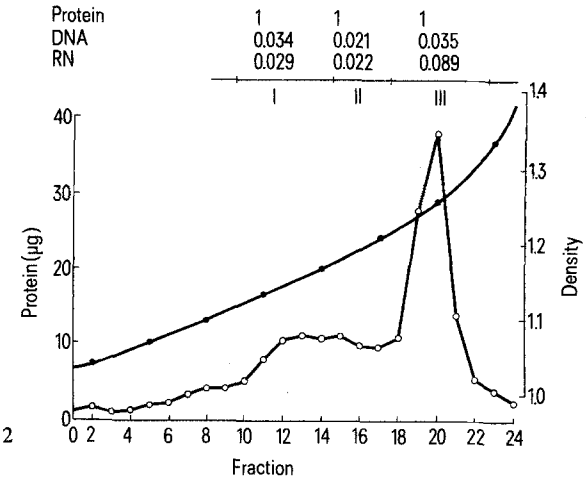

Fig. 2

Fig. 2. Isopycnic metrizamide gradient centrifugation of fraction 2. The samples were layered on preformed 17–58% w/v linear metrizamide density gradients and run for 42 h at 40,000 rpm. Protein[8], DNA[9] and RNA[10] contents were determined in the pooled fractions. ○——○: Protein; ●——● density.

stripped off the nuclei during the Triton treatment, as expected[12], and after the completion of washings the preparations were essentially free of extranuclear debris (data not shown).

We propose, for use in some kinds of experiments, the incubation of nuclei in the low-salt medium, as described above, as a means for the removal of traces of cytoplasmic contaminants from nuclear preparations. Our data reveal that less than 1.8% of the total nuclear DNA is lost during the incubation. It was also demonstrated that the presence of the protease inhibitor, diisopropylfluorophosphate[13], in our incubation system did not result in any change in the amount and composition of fraction 2 liberated. Thus, the extraction is also successful under conditions of efficient inhibition of proteolysis. In our studies[14], use has been made of the described incubation in 2 systems: 1. By employing it before the phosphorylation of nuclear proteins in vitro by [γ-^{32}P]ATP, the total incorporation can be increased because the ATP-ase activity of the nuclear preparations is reduced. 2. By applying it during the purification of a nuclear protein phosphatase, the contamination of the starting material by cytoplasmic phosphatases can be reduced.

Evaluating our data from G-6-P-ase activity measurements, it is apparent that if the incubation of the nuclei in the low-salt medium is not performed, the cytoplasmic contaminants present in fraction 2 will be solubilized with the 0.14 M saline extract. This fact should be considered if one works with the latter.

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Penetration and interaction with haemoglobin of Corynebacteria-like microorganisms into erythrocytes in vitro

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Summary. Following 24 h incubation of normal blood in the presence of the microorganism, the evolution of cell wall deficient forms within the erythrocytes and a process of oxidation of the haemoglobin may be observed.

Previous research by other authors and ourselves has demonstrated within the circulating erythrocytes and platelets the presence of bacterial minimal reproductive units (MRU) and cell wall deficient (CWD) forms, which evolve in the haemocultures towards conventional forms of Corynebacteria-like microorganisms (diphtheroids) and *Staph. epidermidis.* In the majority of cases, the evolution of CWD and the reversion to conventional bacterial forms take place following prolonged periods of incubation[1-4]. No significant differences have been detected between the percentage of growth of cocci and diphteroids within the blood cultures from normal subjects and from patients in various pathological situations. Such growth, in the majority of cases, was accompanied by a more or less pronounced process of haemolysis[5].

The present research has been carried out in order to evaluate the possibility that, by adding a pure culture of a Corynebacterium to sterile blood in vitro, the bacterium penetrates the cell wall of the erythrocyte; and in order to verify the consequences of the multiplication within the erythrocytes of a bacterial strain which, in the primary haemoculture from which it had been isolated, gave rise to a notably strong process of haemolysis.

Materials and methods. The microorganism isolated from the circulating blood of a patient suffering from acute articular rheumatism in a febrile state, has been maintained in our laboratory for about 2 years. Culture media: DIFCO trypticase soy broth and agar, and DIFCO brain heart infusion. The blood agar for the tests of haemolitic activity was prepared with 5% rabbit blood whose sterility was previously checked. For the tests concerning the penetration of the bacteria into the erythrocytes and the alterations of the haemoglobin, 0.2 ml of group 0 human erythrocytes centrifuged at 250 × g 5 min, or 1 ml of the supernatant 4000 × g 10 min of haemolysate prepared from the same erythrocytes resuspended 1:5 in a NaCl 0.25% solution, were mixed with 5 ml of broth. To these preparations 0.2 ml of the bacterial growth of 24 h in broth were added. For the control of the sterility of the blood samples and for the blanks, the bacterial suspension was substituted with 0.2 ml of broth. The suspensions were placed in test-tubes and shaken only at the beginning of the incubation carried out for 24 h at 37 °C. At the end, the cultures with erythrocytes were centrifuged at 400 × g 10 min; the supernatants were discarded and with the sediments were prepared: smears which were stained with Giemsa or acridine orange to recognize the nucleic acids with UV examination; ultrathin sections for the electron microscopy by means of the same methods used for the previous research[3]. Furthermore the erythrocytes were lysed and extensively washed until the ones used as control became completely colourless. They were then suspended in 1 M phosphate buffer pH 6 and spectra were taken from 650 to 470 nm. The products of the incubation carried out in the haemolysate enriched broth were centrifuged at 4000 × g 10 min. On the clear supernatant diluted 1:5 with 1 M phosphate buffer pH 6, spectra were recorded in the visible region.

Results. The physiological characters of the micro-organism used for the present research will be described in a further