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Calcitonin gene-related peptide-1 (CGRP-1) is a potent regulator of glycogen metabolism in rat skeletal muscle

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We investigated the effects of CGRP on glucose metabolism in intact rat skeletal muscle preparations that are largely composed of either type I (soleus) or II fibres (e.g. extensor digitorum longus (EDL) or epitrochlearis muscles). CGRP-1 inhibited insulin-stimulated glycogen synthesis in both soleus and EDL muscle preparations. Rat CGRP-1 was a potent stimulator of glycogenolysis only in muscles composed of type II fibres, which depend on high rates of glycogenolysis to produce high power outputs. These results may provide the basis for understanding how CGRP regulate glycogenolysis in type II fibres in vivo.

Amylin; Insulin resistance; Diabetes mellitus

1. INTRODUCTION

Skeletal muscle is composed of several fibre types. Slow twitch (type I) fibres have a low maximum velocity of shortening (V_{max}) [1] and are specialised for protracted use at relatively low velocities [2]. Fast twitch or type II fibres, which are subclassified as type IIA and IIB, have a high V_{max} [1] and are necessary for maximal rates of locomotion which require large power outputs [3]. Type II fibres have the capacity for high rates of ATP production. This is achieved by increased rates of release of glucosyl units from muscle glycogen (glycogenolysis) and subsequent glycolytic conversion to lactate. Glycogenolytic and glycolytic potential, as measured by maximal enzyme activities, are 2–6-fold higher in type II fibres as compared with type I fibres [4,5]. Adrenaline stimulates glycogenolysis in skeletal muscle via β-adrenoceptors. Surprisingly, responsiveness of muscle glycogenolysis to β-adrenergic stimulation is greater in type I than in type II fibres [6–8]. Noradrenaline has little effect on glycogen metabolism in skeletal muscle [9]. The neuropeptide calcitonin gene-related peptide (CGRP) has 46% primary amino acid sequence identity with the pancreatic peptide amylin [12]. Both peptides inhibit basal and insulin-stimulated glycogen synthesis in incubated stripped soleus muscle preparations [10,13]. Soleus is largely populated with type I fibres. There is evidence for localisation of CGRP in both sensory nerves [14] and in the motor end plate axon terminal [15] in skeletal muscle. In view of the potential neuroendocrine role of this peptide we studied its effects in muscles largely composed of type II fibres (i.e. extensor digitorum longus, EDL, and epitrochlearis). Also, since the EDL muscles were isolated from young rodents the effect of CGRP-1 on intact soleus muscle preparations was also measured.

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2. MATERIALS AND METHODS

2.1. *Materials*

Male Wistar rats were obtained from Harlan Olac (Bicester, England) and were maintained in the Department's animal house. Chemicals and enzymes were obtained from sources

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given in [7,13] except for the cAMP radioimmunoassay kit which was purchased from Amersham.

2.2. *Incubation studies and metabolite assays*

Intact soleus and EDL muscles were prepared from 12-h fasted rats as described previously [16]. The tendons of the muscles were ligated before attachment to stainless steel clips. Muscle strips were pre-incubated in Erlenmeyer flasks containing 3.5 ml Krebs-Ringer bicarbonate buffer plus 7 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, pH 7.4, 5.5 mM glucose, 5 mM pyruvate 5, 1.5% (w/v) de-fatted bovine serum albumin. Flasks were sealed and aerated continuously with O_2:CO_2 (19:1, v/v). After pre-incubation of muscles in this medium for 30 min, at 37°C in an oscillating water bath, the muscle strips were transferred to similar vials containing identical medium (except pyruvate was omitted) with added [U-^{14}C]-glucose (0.5 μCi/ml) and insulin (100 μU/ml). The flasks were sealed, re-gassed for the initial 15 min period in a 1 h-incubation. At the end of the incubation period, muscles were blotted and rapidly frozen in liquid nitrogen. The concentration of lactate in the incubation medium and plasma was determined spectrophotometrically [17] or radiochemically [18] and [U-^{14}C]glucose incorporated into glycogen (glycogen synthesis) was measured [19].

Rates of glycogen synthesis were measured in terms of μmol of glucosyl units$\cdot h^{-1}\cdot g^{-1}$. One glucosyl unit indicates one glucose moiety incorporated into glycogen. For measurement of cAMP levels, muscles were powdered under liquid N_2 before addition of perchloric acid (6%) and the mixture was reground. The concentration of cAMP was measured in neutralised extracts with a radioimmunoassay kit.

3. RESULTS AND DISCUSSION

The effects of various concentrations of rat CGRP-1 on the rates of lactate formation and glycogen synthesis were studied in both the intact soleus and EDL muscle preparations in vitro and the results are shown on fig.1. In both intact EDL and soleus muscles CGRP-1 inhibited the insulin-stimulated rates of glycogen synthesis in a concentration-dependent manner: the CGRP-1 EC_{50} values (i.e. the concentration of hormone necessary for a half-maximal response) for glycogen synthesis were about 0.58 nM and 0.56 nM, respectively. Only the highest concentration (100 nM) of CGRP-1 significantly stimulated the rate of lactate formation in the intact soleus muscle. In marked contrast, CGRP-1, in the presence of insulin, stimulated the rate of lactate formation in a concentration-dependent fashion in the isolated, intact EDL muscle preparation. The calculated EC_{50} value for stimulation of lactate formation was 1.35 nM (if 100 nM CGRP-1 gave the maximal response). Indeed, if the rate of lactate formation is increased at a higher concentration of CGRP-1 then the EC_{50} value from the present results will be an underestimate. CGRP-1 also stimulated lactate formation in isolated incubated epitrochlearis muscle, which is composed of type II fibres (table 1).

In isolated, incubated muscle preparations lactate is formed by glycolytic conversion of glucose molecules that are taken up from the incubation medium (measured as radiochemical lactate formation) or from muscle glycogen [19,20]. In theory, lactate concentration determined spectrophotometrically is a measure of the rate of conversion of glucose derived from both sources. In EDL muscle CGRP-1 stimulated only the rates of spectrophotometric lactate formation (fig.1), but not rates of radiochemical lactate formation (fig.1) or glucose oxidation (results not given); this suggests that CGRP-1 stimulated glycogen breakdown but did not affect glucose uptake. Furthermore, these results suggest that the sensitivity of glycogenolysis to CGRP-1 is markedly decreased in type I fibres in comparison with type II fibres. Phosphorylation of glycogen phosphorylase, which results in rapid breakdown of glycogen, is caused, at least in part, by β-adrenergic agents via a mechanism dependent on cAMP [22,23]. A concentration of CGRP-1 (10 nM), which maximally decreased the rates of glycogen synthesis in both soleus and EDL preparations, increased the levels of cAMP only in EDL muscles (table 2). This would suggest that the mechanism of inhibition of glycogen synthesis by CGRP-1 does not involve cAMP alone. Furthermore, CGRP-1 (1 nM) had no effects on the rates of radiochemical lactate formation in type II fibres incubated in the presence of various concentrations of insulin, but the rates of spectrophotometric lactate formation were significantly increased at all concentrations of insulin (see fig.2).

The soleus muscle of the rat has a post-natal period of maturing, and most of the fast-twitch changes to slow twitch fibres occur over the first few months [24,25]. The intact soleus muscle preparation used in the present study is expected to be populated with 80% type I fibres, the rest being type IIA [24,25]. EDL and epitrochlearis muscle both contain >90% type II fibres (about 50% and 70% type IIB fibres, respectively [26,27]). The chief difference between soleus and other muscles is the presence of type IIB fibres. Our results suggest that CGRP-1 may specifically stimulate

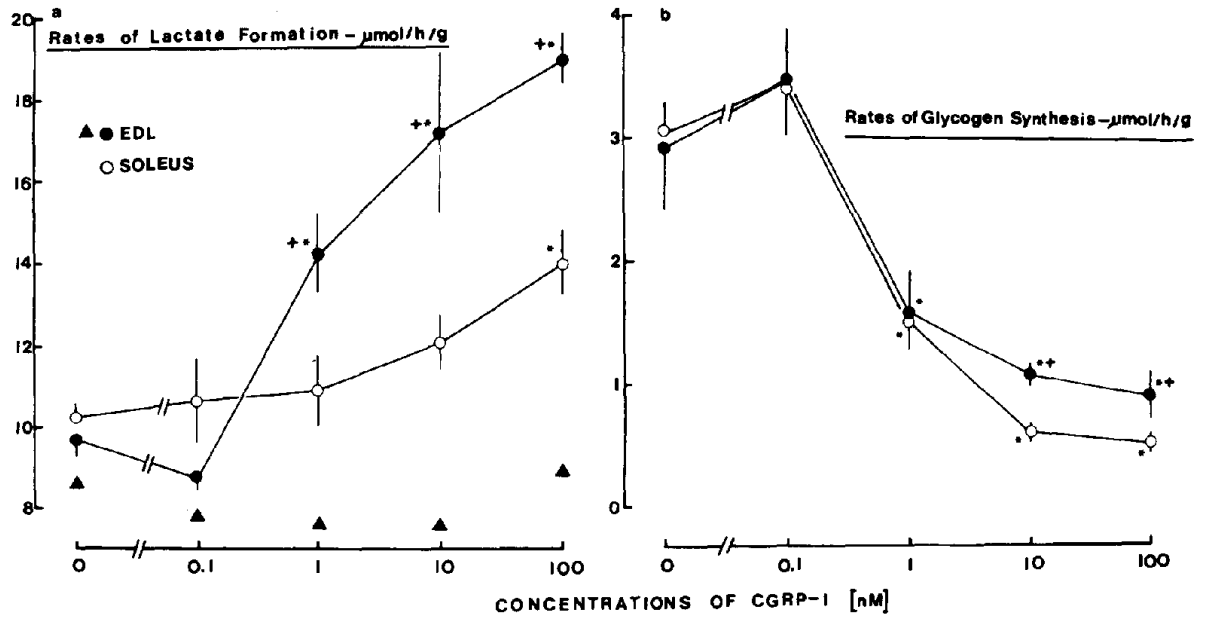

Fig.1. Concentration-dependent effects of rat calcitonin gene-related peptide-1 (CGRP-1) (Sigma) on insulin-stimulated ($\mu U \cdot ml^{-1}$) rates of (a) spectrophotometric lactate formation and (b) glycogen synthesis in isolated incubated intact soleus (○) and extensor digitorum longus (EDL) (●) muscle preparations. The rates of radiochemical lactate formation in incubated EDL muscle preparations are also shown in (▲). The values are presented as means ± standard errors of the means of at least four separate experiments. Statistically significant changes from muscles incubated in the absence of CGRP-1 are indicated by * (Student's *t*-test; $P<0.05$). Statistically significant changes between soleus and EDL muscles at any given concentration of CGRP-1 are indicated by + ($P<0.05$).

glycogenolysis, and hence lactate formation, in type IIB fibres in certain conditions, for example, during high intensity exercise. Quantitatively, the chief site of lactate production is skeletal muscle [28]. Lactate is cleared from the bloodstream mainly by the liver [29,30] by conversion, via gluconeogenesis, to glucose. In type II (non insulin-dependent) diabetes there is a greater flux through the gluconeogenic pathway in both the post-prandial and post-absorptive states [31–33].

Table 1

The effects of rat calcitonin gene-related peptide-1 (CGRP – 1) on insuling-stimulated rates of lactate formation and glycogen synthesis in the incubated epitrochlearis muscle of the rat

Treatment	Rates (μmol/h per g wet wt)	
	Lactate formation	Glycogen synthesis
Control	13.93 ± 0.62	3.95 ± 0.38
CGRP (10 nM)	17.89 ± 1.29*	2.55 ± 0.23*

All results present means ± SE for at least three replicate experiments. Significant differences between control and treated muscles (non-paired Student's *t*-test) are indicated by asterisks, $P<0.05$. Insulin was present in the incubation medium at a concentration of 100 μU/ml

Table 2

Effects of rat calcitonin gene-related peptide-1 (CGRP) on the rates of lactate formation and cyclic AMP content in incubated intact soleus and extensor digitorum longus (EDL) muscle preparations of the rat

	Rates or content ($\mu mol \cdot 10\ min^{-1} \cdot g^{-1}$ or $\mu mol \cdot g^{-1}$)	
	Lactate formation	cAMP content
Soleus		
Control	1.5 ± 0.2 (12)	212 ± 24 (11)
CGRP	1.9 ± 0.1 (12)	255 ± 44 (10)
EDL		
Control	1.5 ± 0.2 (11)	141 ± 18 (11)
CGRP	2.7 × 0.2 (12)*	292 ± 7 (10)*

All results presented as means ± SE for the number of observations given in parentheses. Statistically significant differences (non-paired Student's *t*-test) between control and treated muscles are indicated by asterisks, $P<0.001$

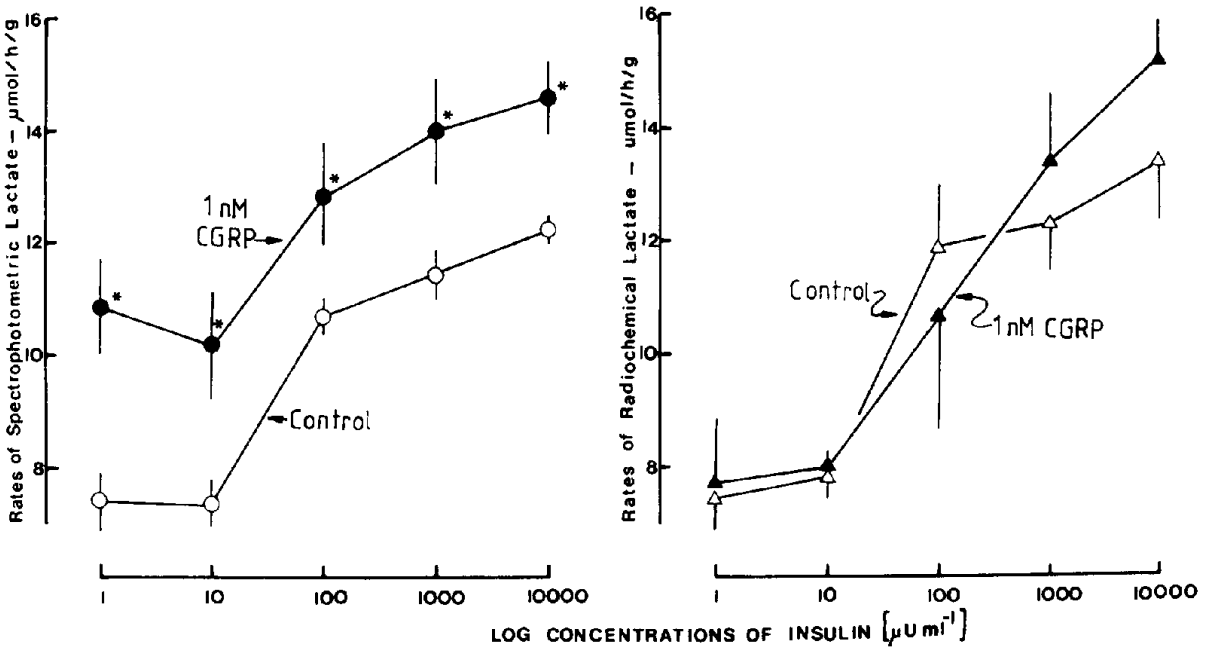

Fig.2. Effects of insulin on the rates of spectrophotometric (○,●) and radiochemical (△,▲) lactate formation in incubated intact extensor digitorum longus muscle preparations of the rat incubated in the presence (●,▲) and absence (○,△) of rat calcitonin gene-related peptide-1. Results are presented as means ± standard errors of the means of at least four individual experiments. Statistically significant differences between (non-paired Student's *t*-test) control (○,△) and muscles treated with rat CGRP-1 (●,▲) are indicated by * ($P<0.05$).

This would be expected to decrease the concentration of lactate in the blood. Paradoxically high blood lactate concentrations are found in type II diabetic subjects [34–36]. This may be explained by higher rates of lactate formation from skeletal muscle which may be caused by higher levels of CGRP and/or amylin [13] and the much higher content of type IIB fibres [37] in insulin resistant subjects. Therefore, the results of the present study are consistent with the hypothesis that an abnormality in CGRP and/or amylin homeostasis underlies the pathogenesis of type II diabetes [13].

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Evidence for a factor promoting the conversion of VWF from low and intermediate to high molecular mass polymers on the platelet membrane

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On the membrane surface of resting platelets exists a factor capable of promoting the conversion of plasma von Willebrand factor from low and intermediate molecular mass to high molecular mass polymers. The process is accompanied by a parallel increase in the von Willebrand factor activity. This could be a regulatory system for the molecular mass distribution of von Willebrand factor during its lifetime in plasma. Glycoprotein Ib of the platelet membrane appears not to be involved in the process.

Hemostasis; Platelet; Von Willebrand factor; Ristocetin

1. INTRODUCTION

The von Willebrand disease is an autosomic coagulation disorder due to a quali- and/or quantitative deficiency of the von Willebrand factor (vWF), a large glycoprotein produced by the endothelial cells and megacaryocytes [1-3]. In plasma, vWF circulates as a heterogeneous mixture of disulfide-bridged polymers ranging from dimers (M_r 25×10^4) to multimers ($M_r > 2 \times 10^7$) and its role is to promote the initial step in hemostatic plug formation [4] by interacting with the subendothelial structures (collagen) of injured vessel and with the glycoprotein IIb-IIIa of activated platelets [5,6]. In the presence of the antibiotic ristocetin, vWF is capable of interacting with resting platelets too: in this case the platelet membrane glycoprotein Ib is responsible for the interaction with the ristocetin-vWF complex [7-11] by which platelets are activated and aggregate. Glycoprotein Ib retains the capability to interact with the ristocetin-vWF complex also when platelets are metabolically inactive such as after treatment with formaldehyde [12,13]; in this case agglutination of FFP occurs.

Both the physiological interaction of vWF with activated platelets, and the non-physiological interaction of the ristocetin-vWF complex with resting or inactivated platelets, strongly depend on the molecular mass of the polymers, the highest activity being shown by the largest molecules.

A lot of information exists indicating that the vWF molecules produced by the endothelial cells are stored into the Weibel-Palade bodies as a mixture containing all the polymeric forms, but it is not known, to date, whether this is the only way by which the HMW polymers are produced, or whether polymerization may also occur after the secretion of glycoprotein into blood [14-16]. The problem becomes more puzzling because of the recent observation [17] that the proteolytic cleavage of the precursor (pro-vWF) seems not to be essential for the multimers formation.

In the present paper we show that on the resting platelet membrane surface there is a factor capable of promoting the conversion of vWF from low and intermediate molecular mass to high molecular mass polymers with a consequent increase in the vWF activity.

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2. MATERIALS AND METHODS

2.1 *Materials*

All reagents were analytical grade. Human platelet poor plasma (PPP) was prepared by double centrifugation (2000×*g*; 15 min) of citrate (0.38%) added to fresh blood samples from volunteers. Platelet rich plasma (PRP) was obtained by double centrifugation of citrate added to blood (200×*g*; 8 min). Washed platelets (WP) were prepared immediately before use by centrifugation of PRP (3000×*g*; 15 min) followed by three washing cycles of the pellet with 10 mM Tris, 1 mM EDTA, pH 7.5, buffer (Tris-EDTA buffer): from 40 ml of fresh blood we usually obtained 10 ml of washed platelets ($\sim 4\times10^5$ WP/μl). Formaldehyde-fixed platelets (FFP) were prepared by one hour preincubation of PRP suspensions (10 ml) at 37°C, followed by centrifugation (2500×*g*; 15 min) and resuspension of the pellet in 10 ml of Tris-EDTA buffer containing 2% formaldehyde; after 48 h incubation at 4°C the suspension was centrifuged (2500×*g*; 15 min) and the pellet was resuspended in Tris-EDTA buffer giving 3×10^5 FFP/μl (10–12 ml) and stored at 4°C. Incubations of PPP samples with FFP or WP were performed by centrifuging (3000×*g*; 15 min) the desired amount of platelets from the storage buffer and gently resuspending the pellet in the required volume of plasma to be treated.

The experiments requiring WP were preceded by a control on the integrity of platelets by suspending them (3×10^8 WP/ml) in 200 μl of PPP in the aggregometer vessel and following the aggregation process induced by 2 μM ADP. Parallel to these experiments, aggregometric controls were routinely performed in order to check whether incidental activation of WP occurred during incubations.

2.2. *vWF activity in plasma*

200 μl of FFP suspension (3×10^5 FFP/μl), supplemented with 1 mg/ml ristocetin, were preincubated 3 min at 37°C into the aggregometer vessel under stirring, after which 50 μl of the PPP to be tested were added; the aggregometer (Menarini) recorded the increase in transmittance, due to the agglutination of platelets, vs time. The slope of the initial agglutination time course was proportional to the activity of the vWF in the sample. We assumed 100% activity to be that obtained for a PPP sample from a pool of blood samples from voluntary donors under the indicated test conditions.

2.3 *Agarose gel electrophoresis, transblotting, staining*

Gel electrophoresis was performed substantially as in [18] with some modifications. Gels, 1 mm thick, were 10×10 cm size, the stacking and the running parts were 0.75% and 1.75% agarose and were 2.5 cm and 7.5 cm in length, respectively; sample wells (2×0.2 cm) were cut in the stacking gel at 1 cm from the interface. Plasma to be investigated was diluted 5-fold with Tris-EDTA buffer adjusted to pH 8.0 and containing 2% SDS, 8 M urea and 0.01% bromophenol blue as tracking dye. After 20 min incubation at 60°C, 20 μl samples were applied to each well and electrophoresis started at 20 mA constant current. After the samples had moved out of the wells, these were filled with stacking gel suspension and the current was decreased to 4 mA. Electrophoresis was stopped when the dye reached the anode end of the gel (usually 14 h). After the run the gel was transblotted onto a nitrocellulose sheet (Bio-Rad, 10×10 cm, 0.22 μm) by a standard transblot chamber (Bio-Rad) and following the recommended procedure (24 h run; 200 mA constant current; 4°C temperature; running buffer, 15.6 mM Tris, 120 mM glycine, pH 8.3, containing 0.05% SDS, 20% methanol). The nitrocellulose sheet was treated (30 min) with fixing buffer (2-propanol/acetic acid/water, 25:10:65), washed three times with Tris-NaCl buffer (50 mM Tris, 150 mM NaCl, pH 7.5), saturated by incubation (30 min) in Tris-NaCl buffer containing 1% bovine serum albumin and washed three times with Tris-NaCl buffer containing 1% Triton X-100. Staining was obtained by a 4 h incubation of the nitrocellulose sheet with peroxidase-conjugated anti-human vWF rabbit antiserum (DAKO) diluted 1:125 with Tris-NaCl buffer containing 1% Triton X-100, followed by four washing cycles with the same buffer and four washing cycles with Tris-NaCl buffer. The last washing bath was supplemented, in the dark, with 0.5 mg/ml 4-chloro-1-naphthol (predissolved in methanol) and 5 mM hydrogen peroxide. The incubation was stopped after 20 min by repeated washings with distilled water. After air drying, the nitrocellulose sheet was photographed.

3. RESULTS AND DISCUSSION

A typical PPP from healthy donors shows the molecular mass distribution of vWF polymers represented in fig.1 (lane 1), the activity (as defined in section 2.2) of which was taken as 100%. In the presence of ristocetin and FFP, a ristocetin-vWF complex was formed capable of interacting with the membrane glycoprotein Ib of FFP [12,13] by which agglutination occurred and, consequently, the removal of vWF bound to platelets from the medium was possible by centrifugation. As shown in lane 2, by an agglutination–centrifugation treatment, the high molecular mass vWF polymers (HMW-vWF) were removed from plasma with a drop of activity to 25% with respect to sample 1. The intermediate molecular mass polymers (IMW-vWF) were removed from plasma by a second identical treatment by which only the low molecular mass polymers (LMW-vWF, i.e. dimers and tetramers) remained in the medium with an activity near zero (lane 3).

A PPP sample (fig.2, lane 1), subjected to an agglutination–centrifugation treatment to remove the HMW-vWF polymers, was incubated with WP and subsequently centrifuged. In the supernatant obtained after an hour of incubation (lane 3), the trace corresponding to the highest polymer of lane 2 completely disappeared and, after two hours of incubation, a sharp decrease in the intensity of all traces was observed. This result is interpreted as a consequence of the concurrent effects of WP and