résultats obtenus avec le milieu SB-BHI (tableau 2), ils sont comparables à ceux obtenus en milieu TSB. Ainsi, on ne note aucun effet du ribonucléate de sodium sur la croissance des souches ATCC 27164, PM_9 et PA_2 et, alors que l'activité hémolytique de la souche ATCC 27164 augmente d'un facteur de 100 en présence de 1% de ribonucléate de sodium, dans le cas des souches PM_9 et PA_2 ce facteur est de 40. Enfin, il faut noter que les maxima d'activité dans le cas des souches PM_9 et PA_2 se situent au 4e jour comparativement au 3e dans le cas du milieu TSB. Dans les 2 milieux l'utilisation de ribonucléate à une concentration de 1,5% inhibe complètement la croissance. Il appert donc de ces résultats que les milieux TSB et SB-BHI se comparent avantageusement pour la croissance des différentes souches de tréponèmes et que le ribonucléate de sodium à une concentration de 1% permet d'augmenter considérablement aussi bien l'activité hémolytique des souches fortement β-hémolytiques que des faiblement β-hémolytiques. L'effet du ribonucléate de sodium sur la production de l'activité hémolytique par ces souches n'est pas sans rappeler celui observé dans le cas de la streptolysine S des streptocoques du groupe A[7] et de l'aérolysine[8]. Si on ne connaît pas encore le rôle, au niveau moléculaire, de ce composé vis-à-vis l'aérolysine, on sait, par contre, que dans le cas de la streptolysine S, il agirait en premier lieu en libérant cette toxine de la surface des cellules et lui servirait ensuite de transporteur[7]. Des essais préliminaires ont démontré que l'addition de ribonucléate de sodium au surnageant d'une culture de *T. hyodysenteriae* ATCC 27164 effectuée en absence de ce composé, n'entraîne aucune augmentation de l'activité hémolytique ni de sa stabilité à la température. Ces observations, jointes à celle du rendement cellulaire inchangé des cultures en présence de ribonucléate de sodium, posent la question du mécanisme par lequel ce composé entraînerait l'augmentation de l'activité hémolytique de ces tréponèmes.

1 A qui toute correspondance doit être adressée.
2 R.D. Glock et D.L. Harris, Vet. Med., Sm. Anim. Clin. *67*, 65 (1972).
3 D.L. Harris, R.D. Glock, C.R. Christensen et J.M. Kinyon, Vet. Med., Sm. Anim. Clin. *67*, 61 (1972).
4 D.J. Taylor et T.J.L. Alexander, Br. vet. J. *127*, 58 (1971).
5 M.J. Hudson, T.J.L. Alexander et R.J. Lysons, Vet. Rec. *98*, 498 (1976).
6 J.M. Kinyon, D.L. Harris et R.D. Glock, Infect. Immun. *15*, 638 (1977).
7 L. Ginsburg, in: Microbial Toxins, vol. IV, p.99. Academic Press, New York 1970.
8 A.W. Bernheimer et L.S. Avigad, Infect. Immun. *9*, 1016 (1974).
9 J.G. Songer, J.M. Kinyon et D.L. Harris, J. clin. Microbiol. *4*, 57 (1976).
10 J.M. Kinyon et D.L. Harris, Vet. Rec. *95*, 219 (1974).
11 S.A. Saheb et R. Richer-Massicotte, Rev. Can. Biol. *31*, 153 (1972).
12 G.G. Meynell et E. Meynell, in: Theory and practice in experimental bacteriology, 2nd ed., p. 19. Cambridge University Press 1970.
13 A.W. Bernheimer et L.L. Schwartz, J. gen. Microbiol. *30*, 455 (1963).
14 L.F. Cooper, M.A. Madoff et L. Weinstein, J. Bact. *87*, 127 (1964).

Increased activity of the ribosomal dissociation factor in the pre-replicative phase of liver regeneration after partial hepatectomy

R. Comolli, A. Schubert, M. Cojazzi and L. Riboni[1]

Centro di Studio per la Patologia cellulare del C.N.R. and Istituto di Patologia generale, Università di Milano, via Mangiagalli, 31, I-20133 Milano (Italy), 19 July 1978

Summary. The activity of the ribosomal dissociation factor and the formation in vitro of free 60S and 40S subunits increased in the first 12–48 h after partial hepatectomy. This suggests an accelerated reconversion into active subunits of ribosomes that complete a translation cycle in the early phases of liver regeneration.

Following the partial surgical removal of rat liver, a decreased rate of protein degradation might account for at least part of the increased rate of protein accumulation in this condition[2]. The liver shows an increased amino acid incorporation into protein during regeneration[3, 4] and the ribosomes occur mainly in the form of polyribosomes[5–7]. In conditions of accelerated synthesis of proteins, the activity of the soluble protein factors involved in the initiation reactions of polypeptide chain formation is found to increase[8–10]. One of the protein factors, the eIF-3 (or DF) factor[11, 12], prevents the association of 60S and 40S subunits into 80S monomers and promotes 80S monomer ribosome dissociation into subunits[11–16].

The dissociation of ribosome monomers into subunits is a prerequisite for the initiation of protein synthesis[17, 18]. In the present work we have studied the activity of this factor, extracted by high-salt wash from ribosomes and from the cell sap of rat liver, at 12, 24, 48 and 72 h after partial hepatectomy and sham-operation and in intact control rats of same age.

Material and methods. Male Wistar rats weighing 250–300 g (5–6 months old) fed ad libitum until death and housed with fixed artificial illumination from 07.00 to 19.00 h, were used. 4–6 animals per experiment were submitted to partial hepatectomy (67%)[19] between 09.30–11.00 h; 2 animals per experiment were employed for sham-operation and palpation of the liver. Rats were killed by cervical dislocation and bled. Control rats were killed at the same day period (09.30–11.00 h). The livers were excised, weighed and homogenized at 0–4 °C in 20 mM tris-Cl buffer, pH 7.6, 100 mM NH_4Cl, 5 mM Mg acetate, 2 mM mercaptoethanol (MSH), 0.2 M sucrose. Ribosomes and the cell sap were obtained and purified by conventional methods[9, 20, 21].

The extraction of the dissociation factor from ribosomes and from the cell sap was as reported previously[20, 22]. Ribosomes were suspended in high-salt medium (10 mM tris-Cl buffer, pH 7.6, 0.5 M KCl, 10 mM MSH) and the ribosomal protein containing the factor extracted. The KCl extract was centrifuged at 100,000 × g, brought to 30% (40% for cell sap) saturation with ammonium sulphate and then to 70% saturation. The 70% precipitate was suspended in 10 mM tris-Cl buffer, pH 7.6, 100 mM KCl, 0,3 mM Mg acetate, 2 mM MSH, dialyzed against this buffer and applied to a 1.5 × 10 cm DEAE-cellulose (Whatman DE 23) column, equilibrated with this buffer with KCl increased to 120 mM. The factor activity was eluted with the

Dissociation factor activity of the liver cell extracts of partially hepatectomized, sham-operated and intact control rats

	Hepatectomized				Sham-operated				Controls
Period of regeneration (h)	12 (5)	24 (4)	48 (3)	72 (3)	12 (5)	24 (4)	48 (3)	72 (3)	(6)
Ribosomal high-salt wash									
100 µg protein	0.064 ± 0.0148	0.098 ± 0.0269	0.165 ± 0.0385	0.079 ± 0.0188	0.029 ± 0.0220	0.160 ± 0.0271	0.137 ± 0.0420	0.020 ± 0.0129	0.055 ± 0.0141
200 µg protein	0.140 ± 0.0197	0.202 ± 0.0161	0.244 ± 0.0245	0.129 ± 0.0148	0.053 ± 0.0198	0.211 ± 0.0201	0.194 ± 0.0291	0.026 ± 0.0061	0.123 ± 0.0133
Cell sap									
100 µg protein	0.054 ± 0.0264	0.131 ± 0.0340	0.096 ± 0.0299	0.071 ± 0.0278	0.015 ± 0.0120	0.033 ± 0.0189	0.187 ± 0.0397	0.031 ± 0.0215	0.040 ± 0.0164
200 µg protein	0.096 ± 0.0295	0.293 ± 0.0459	0.227 ± 0.0264	0.100 ± 0.0251	0.018 ± 0.0057	0.098 ± 0.0268	0.246 ± 0.0352	0.052 ± 0.0256	0.110 ± 0.0173

The concentration of the extracts was 100 and 200 µg protein and the spontaneous dissociation during the incubation was subtracted. Means ± SE. The number of experiments is given in parenthesis

same buffer containing 300 mM KCl. The protein concentration of the eluate was measured by the Lowry method[23] or estimated by UV absorption[22].

The assay of the dissociating activity was performed[22, 24] at 25 °C for 15 min on liver ribosomes pretreated with puromycin[25] and suspended in 10 mM tris-Cl buffer, pH 7.6, 100 mM KCl, 0.3 mM Mg acetate, 5 mM MSH, 0.3 mg/ml bovine albumin (fraction V)[26]. After incubation, the samples were fixed with glutaraldehyde (1:2) 2.5% final concentration[26] and centrifuged at 24,000 rpm in a Spinco SW 27 rotor for 270 min over 15-30% linear sucrose gradients in the same buffer. The gradients were monitored at 260 nm[20] and the absorbance profiles recorded and quantitated directly by cutting out the peaks and weighing the paper. Measures were repeated for reproducibility and the dissociation determined as follows: dissociation ratio = (40S + 60S peak area)/(40S + 60S + 80S peak area).

Results and discussion. The results are reported in the table. The procedure of operation produced a transient fall of factor activity in the first hours post-operation that was more evident in sham-operated than in partially hepatectomized rats. After hepatectomy the activity of the ribosomal wash factor increased at 12, 24 and 48 h post-operation. The activity of the cell sap factor increased at 24 and also 48 h. In sham-operated rats the activity of the dissociation factor was reduced at 12 h. Then the activity showed an increase: at 24 and 48 h in the ribosomal wash extracts and at 48 h in the cell sap. This might reflect the increased synthesis of fibrinogen and other blood proteins in this condition[27], due to the effects of injury. At 72 h after operation the dissociating activity declined. However, the decline was greater in sham-operated rats.

These observations suggest a precocious increased activity and binding of the dissociation factor to the native ribosomal subunits[11] during the early pre-replicative phases of liver regeneration after hepatectomy[28], that is then followed by an increased activity of cell sap. This might result in an accelerated reconversion into active 40S and 60S subunits of ribosomes that complete a translation cycle[18]. An increased formation of 40S and then 80S initiation complexes[16] might therefore characterize the early phases of the growth of rat liver after partial hepatectomy.

1 The technical assistance of Miss M. Ravazzani and Miss L. Zingaretti is gratefully acknowledged.

2 O.A. Scornik and V. Botbol, J. biol. Chem. *251*, 2891 (1976).
3 C. Majumdar, K. Tsukada and I. Lieberman, J. biol. Chem. *242*, 700 (1967).
4 K. Tsukada, T. Moriyama, T. Umeda and I. Lieberman, J. biol. Chem. *243*, 1160 (1968).
5 P. Cammarano, G. Giudice and B. Lukes, Biochem. biophys. Res. Commun. *19*, 487 (1965).
6 T.E. Webb, G. Blobel and V.R. Potter, Cancer Res. *26*, 253 (1966).
7 A.J. Rizzo and T.E. Webb, Biochim. biophys. Acta *169*, 163 (1968).
8 J. Hopkinson, P.M. Prichard and E. Bresnick, Biochim. biophys. Acta *374*, 375 (1974).
9 C.N. Murty, E. Verney and H. Sidransky, Biochem. J. *152*, 143 (1975).
10 D.M. Nicholls, J. Carey and W. Sendecki, Biochem. J. *166*, 462 (1977).
11 H.A. Thompson, I. Sadnik, J. Scheinbuks and K. Moldave, Biochemistry *16*, 2221 (1977).
12 W.F. Anderson, Ann. N.Y. Acad. Sci. *241*, 142 (1974).
13 M. Ayuso-Parilla, E.C. Henshaw and C.A. Hirsch, J. biol. Chem. *248*, 4386 (1973).
14 K. Nakaya, R.S. Ranu and I.G. Wool, Biochem. biophys. Res. Commun. *59*, 237 (1973).
15 M. Gottlieb, B.D. Davis and R.C. Thompson, Proc. nat. Acad. Sci. USA *72*, 4238 (1975).
16 H. Weissbach and S. Ochoa, A. Rev. Biochem. *45*, 191 (1976).

17 G.A. Howard, S.D. Adamson and E. Herbert, J. biol. Chem. *245*, 6237 (1970).
18 E.C. Henshaw, D.G. Guiney and C.A. Hirsch, J. biol. Chem. *248*, 4367 (1973).
19 G.M. Higgins and R.M. Anderson, Archs Path. *12*, 186 (1931).
20 R. Comolli, A. Carrara Schubert and C. Delpiano, Exp. Geront. *12*, 89 (1977).
21 D.P. Leader and I.G. Wool, Meth. Enzym. *30*, 180 (1974).
22 M. Decroly and M. Goldfinger, Biochim. biophys. Acta *390*, 82 (1975).
23 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
24 M. Gottlieb, N.H. Lubsen and B.D. Davis, Meth. Enzym. *30*, 87 (1974).
25 G. Blobel and D. Sabatini, Proc. nat. Acad. Sci. USA *68*, 390 (1971).
26 A.R. Subramanian, Biochemistry *11*, 2710 (1972).
27 I.M. Murray-Lyon, H.H. Liem and U. Muller Eberhard, Br. J. exp. Path. *56*, 247 (1975).
28 J.I. Fabrikant, J. Cell Biol. *36*, 551 (1968).

The detection of superoxide anion from the reaction of oxyhemoglobin and phenylhydrazine using EPR spectroscopy[1]

B. Goldberg[2], A. Stern[3], J. Peisach and W.E. Blumberg

Department of Pharmacology, New York University School of Medicine, New York (N.Y. 10016, USA), Departments of Molecular Pharmacology and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx (N.Y. 10461, USA), and Bell Laboratories, Inc., Murray Hill (New Jersey 07974, USA), 3 July 1978

Summary. The low temperature EPR spectrum of a quickly reacted mixture of oxyhemoglobin and phenylhydrazine was studied. With the use of a computer, the spectral contribution of methemoglobin in the region of $g=2$ was subtracted. The remaining spectrum was that of an axial free radical ($g_{\perp}=2.00$, $g_{\parallel}=2.06$) having the magnetic parameters of superoxide anion. In the presence of superoxide dismutase, this axial radical was not seen, confirming that superoxide anion is indeed generated by the reaction.

It has been shown by chemical means[4] that the interaction of phenylhydrazine with HbO_2 leads to the generation of superoxide anion, O_2^-, a partially reduced form of molecular oxygen. Rather than being derived from the bound oxygen of hemoglobin, it was found that O_2^- arises from the reaction of molecular O_2 with phenyldiazine, a partially oxidized form of phenylhydrazine which is produced as the primary reaction product of HbO_2 and phenylhydrazine[5].

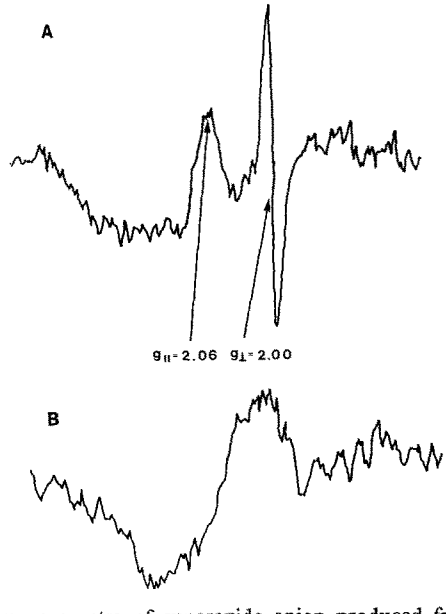
Although superoxide anion has a single unpaired spin, its reactivity in aqueous solution prevents an accumulation of this species in sufficiently reasonable concentration so that it can be detected by conventional EPR methods. It is possible though, to use stopped flow techniques in order to physically demonstrate the formation of this species.

In this present study, we demonstrate for the first time, using a combination of stopped flow and computer facilitated difference EPR techniques, the formation of superoxide anion in a reaction mixture containing HbO_2 and phenylhydrazine. We also show that in the presence of superoxide dismutase, O_2^- does not accumulate so that the EPR spectrum characteristic of this species is not seen.

Materials and methods. Oxyhemoglobin was prepared from a red cell lysate by a standard procedure. For the EPR study, 2 solutions were prepared. The first consisted of HbO_2 (0.8 mM) in 50 mM Na-PO_4 buffer, pH 7.0, containing EDTA (100 µM). The second, phenylhydrazine (3.2×10^{-3} M) in the same aerated buffer. The solutions were individually transferred to 5 ml hypodermic syringes which were components of a manually operated stopped flow apparatus. The syringe effluents were simultaneously combined, mixed in a Gibson-Durrum type mixing chamber and squirted into an EPR cavity[6] precooled in liquid N_2.

EPR spectra were taken at 1.4° on an X-band spectrometer described by Feher[7] operating near 9200 mc/sec and with a sweep rate of 1000 G per 2.5 min. Data obtained near $g=2$ in the first 1.6 min of the sweep were digitized as 1024 data points and were stored for computer processing. The spectrum in the region of $g=6$ was also recorded. The EPR spectrum of an oxyhemoglobin solution to which buffer was added was obtained in the same way. Similar experiments were performed using HbO_2 solutions as above, but to which 0.143 mg/ml of bovine superoxide dismutase (Truett Laboratories) was added.

Results and discussion. Due to the short lifetime of O_2^- in aqueous solution, EPR examination of this species is performed in the frozen state. Using a technique of rapid mixing of reagents followed by fast freezing, it has been possible to examine the EPR spectrum of this species[8]. The experiments described in this present work concerning O_2^-



EPR difference spectra of superoxide anion produced from the reaction of oxyhemoglobin and phenylhydrazine. Spectra were obtained by rapid mixing of reagents followed by quick freezing in an EPR cavity. In A, the spectrum was recorded after phenylhydrazine addition and from which the EPR spectral contribution of methemoglobin was subtracted. In B, the same procedure as in A was followed, but in the presence of superoxide dismutase. The broad, unresolved feature observed to lower field of $g=2$ arises from Cu(II) in the superoxide dismutase.