Biochemical Pharmacology, Vol. 20, pp. 2065–2070. Pergamon Press. 1971. Printed in Great Britain

PROTECTION BY GLUTATHIONE AND PROPYL GALLATE ON THE IMPAIRED *IN VITRO* AMINO ACID INCORPORATION INTO LIVER MICROSOMAL PROTEIN OF CCl_4-POISONED RATS

ENRICO GRAVELA, LUDOVICA GABRIEL and GIANCARLO UGAZIO

Istituto di Patologia Generale dell'Università di Torino, Turin, Italy

(*Received* 7 *December* 1970; *accepted* 2 *February* 1971)

Abstract—The endogenous mRNA directed amino acid incorporation into rat liver microsomes *in vitro* is lowered as soon as 30 min after CCl_4 poisoning; at the same time a strong decrease in rat liver polysomes appears. Previous treatment with the watersoluble antioxidants glutathione and propyl gallate completely prevents these changes in polysome activity and structure. These results seem to indicate a direct relationship between lipoperoxidation and alteration in protein synthesis.

VERY early after CCl_4 poisoning the unsaturated lipids of liver cell microsomes are believed to be attacked by trichloromethyl free radicals and undergo peroxidative decomposition.[1,2] In addition, the rate of amino acid incorporation into both liver protein and liver microsomes is lowered within less than 1 hr after intoxication.[3] Analysis of the sedimentation pattern revealed a loss of hepatic polysomes with a concomitant increase in the amount of both monomeric ribosomes and ribosomal sub-units. Smuckler and Benditt[3] concluded that the impairment of protein synthesis is accounted for by an alteration of the ribosomes, rather than of the enzymatic activities involved in the reaction. Mager *et al.*[4] found that the amino acid incorporation directed by endogenous mRNA was decreased, while at the same time poly(U)-directed phenylalanine incorporation was increased. Hence, these investigators suggested that the decreased *in vitro* uptake of amino acids by liver microsomes of CCl_4-treated rats can be due to the exhaustion of mRNA. Weksler and Gelboin[5] confirmed these results and demonstrated that liver microsomes obtained from rats 2 hr after oral administration of CCl_4 (200 μl per 100 g body wt.) behave like microsomes of control rats, made devoid of mRNA activity by previous incubation at 37°.

The peroxidation of liver lipids initiated by CCl_4 may possibly challenge the GSH-peroxidase system, which has been reported to detoxicate the hydrolipoperoxides present in the cell.[6–10] In addition, the SH-dependent enzymes operating in protein synthesis may be susceptible to peroxidative attack.[11] Nevertheless, a direct interrelationship between lipoperoxidation and decreased protein synthesis has not been evidenced. On the contrary, Alpers *et al.*[12] have questioned a possible role played by lipoperoxidation, since neither DPPD* nor vitamin E prevent the structural and functional derangement of liver ribosomes induced by CCl_4 feeding.

* Abbreviations used: DPPD = *N-N'*-diphenyl-*p*-phenylenediamine.

Recent work in our laboratory[13] has shown than *in vivo* treatment with either DPPD or hydrosoluble antioxidants, namely glutathione or propyl gallate, protect liver polysomes from the changes in sedimentation pattern observed very early after poisoning. Therefore, it seemed worthwhile to examine also the rate of amino acid incorporation into liver microsomes of rats protected with either glutathione or propyl gallate. A preliminary report on part of these results has been presented elsewhere.[14]

MATERIALS AND METHODS

Animal treatment. Female rats, of the Sprague–Dawley strain, in the body weight range of 200–250 g, were starved for 15–18 hr before intoxication. The animals had free access to water. When an antioxidant was used, 80 mg glutathione (DE.BI., Milano, Italy) or 30 mg propyl gallate (C.Erba, Milano, Italy) per 100 g body wt. were injected as a water solution into the peritoneal cavity 30 min before poisoning. Control animals received an equal amount of saline. CCl_4, 250 μl per 100 g body wt., was given by stomach tube as a mixture with mineral oil (1 : 1, v/v). The animals were sacrificed 30 min after poisoning.

Microsome preparation. 20% liver homogenates (w/v) were prepared with a Potter-Elvehjem apparatus, fitted with a Teflon pestle, in a medium (TKM buffer) containing 0·15 M sucrose, 50 mM tris–HCl buffer pH 7·8, 25 mM KCl and 5 mM $MgCl_2$. The postmitochondrial supernatant (S_2) was prepared by centrifuging the homogenate at 15,000 *g* for 10 min. Microsomes and cell sap were harvested by centrifuging the S_2 at 150,000 *g* for 40 min (preparative ultracentrifuge, Beckman-Spinco, model HV, rotor 50). The microsomal pellet was resuspended in TKM buffer with manual homogenization and diluted to a final concentration of 200 mg equiv./ml.

Incorporation of amino acid into microsomes. The incubation medium contained tris–HCl buffer, KCl and $MgCl_2$ as in TKM buffer; 2 mM ATP, 10 mM Phosphocreatine, 0·25 mM GTP, 25 μg creatine phosphokinase, 20 mg equiv. microsomes, 20 mg equiv. cell sap (obtained from untreated animals). Final volume was 0·5 ml. In the [^{14}C]amino acid incorporation study 0·05 μc of [^{14}C]protein hydrolysate from Chlorella (specific activity 52 mc/mAtom carbon; The Radiochemical Centre, Amersham, England) were added at zero-time. The mixture was incubated at 37° in a Dubnoff apparatus for 2, 4 and 6 min, and then treated with 5% trichloroacetic acid (TCA). In the phenylalanine incorporation study, carried out either in the presence or in the absence of poly(U) (final concentration 100 μg per ml), 0·1 μc of (U) [^{14}C] phenylalanine (specific activity 504 mc/m-mole; The Radiochemical Centre, Amersham, England) was diluted with carrier phenylalanine in order to achieve a final concentration of 5 μM phenylalanine in the incubation medium. The labeled phenylalanine was added at the zero-time. The mixture was incubated at 37° for 15 min, and then treated with 5% TCA. The precipitate was incubated at 90° for 20 min in 5% TCA and then treated with a procedure similar to that reported by Stahl *et al.*[15] The protein-bound radioactivity was counted with a liquid scintillation system (L.S. spectrometer, model mark-I, Nuclear Chicago Corp.). The radioactivity levels were corrected, after subtraction of the zero-time control counts, by the counting efficiency (channel-ratio method), and referred to the microsomal protein. An aliquot of the TCA-precipitate was assayed for protein content by means of the method of Lowry *et al.*[16]; bovine serum albumin was used as a standard.

Sedimentation pattern. 0·2 ml of postmitochondrial supernatant, previously treated

with sodium deoxycholate (9 parts S_2 added with 1 part of 10% deoxycholate), were layered over 5·5 ml exponential gradients of 0·5–1·5 M sucrose in TKM buffer, prepared according to a method similar to that reported by Henderson[17] with a 2·5 ml mixing chamber. The gradients were centrifuged at 204,000 *g* for 40 min (ultracentrifuge Beckman-Spinco, model HV, rotor SW 50) at 4°, and then monitored at 254 nm with an apparatus ISCO-UV2 model D, provided with a flow cell having a 2 mm light-path.

RESULTS AND DISCUSSION

Figure 1 shows the time sequence of [^{14}C]amino acid incorporation into microsomes of normal and CCl_4-poisoned rats, either in the absence (left) or in the presence (right) of *in vivo* administered glutathione. The decrease in incorporation rate due to CCl_4 intoxication is highly significant at all the experimental points. By using the [^{14}C] protein hydrolysate from Chlorella, the amino acid uptake by microsomes is not linear during the incubation in our experimental conditions. Nevertheless the per cent of depression in specific activity is constant (36 per cent) within the range taken into

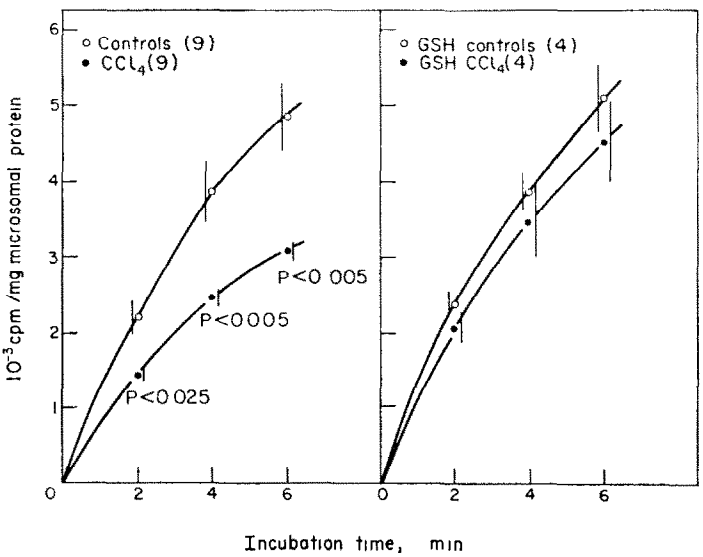

FIG. 1. *In vitro* incorporation of [^{14}C]protein hydrolysate into microsomal protein from control and CCl_4-poisoned rats treated in the presence (right) or in the absence (left) of glutathione. Each point represents the mean from a number of animals (in parenthesis) ± that standard error (vertical bars).

consideration in our experiments, and it is very close to the corresponding values reported by Alpers and Isselbacher[18] for the same experimental period. The preliminary treatment with glutathione restored the lowered incorporation rate to the control level. No detectable effect on control animals has been elicited by GSH alone.

In the second series of experiments (Fig. 2), [^{14}C]phenylalanine was used as tracer amino acid. In our experimental conditions the rate of incorporation into microsomes is linear up to 20 min of incubation, while the specific activity of microsomal protein is lower than that obtained by using [^{14}C]protein hydrolysate. When no poly(U) was added to incubation medium, the phenylalanine incorporation directed by endogenous

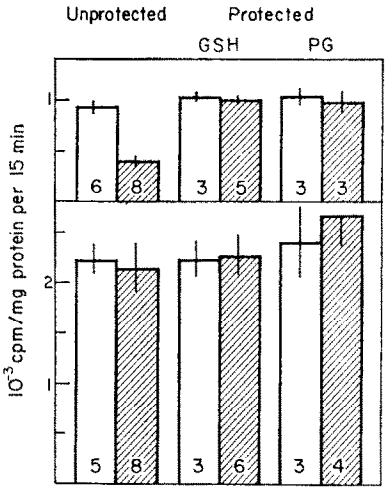

FIG. 2. [^{14}C]phenylalanine incorporation into the microsomal protein of rats poisoned with CCl_4 30 min after treatment with either glutathione or propyl gallate. Upper and lower part of the graph represent the incubation system either devoid of or provided with poly(U), respectively. The height of the blocks represents the mean from a number of animals (reported on the blocks); the values are counts/min/mg of protein/15 min, ± the standard error (vertical bars). Open blocks: control animals. Filled blocks: poisoned animals.

mRNA is lowered by CCl_4 poisoning (62 per cent inhibition). Previous treatment with either glutathione or propyl gallate completely prevents this alteration of protein synthesis.

In some experiments, the sedimentation pattern of ribosomes was studied in association with amino acid incorporation. The results suggest the presence of a direct relationship between the alteration of the ultracentrifugal pattern and the depression of incorporation. In a typical experiment (Fig. 3) the highest dissociation of polysomes (dotted line) corresponds with the lowest specific activity (160 counts/min/mg/15 min), while the slightly affected polysomes incorporate phenylalanine at higher rate (dashed line: 580 counts/min/mg/15 min), and particles from control animals (solid line) show the maximal specific activity (1090 counts/min/mg/15 min). In addition when CCl_4 poisoning follows either glutathione or propyl gallate treatment, the protection of *in vitro* amino acid incorporation into microsomal protein, observed in this research, fits with an unaffected polysomal pattern.

When the incubation medium contained poly(U), no depression of incorporation rate was observed in the CCl_4-poisoned animals and protein specific activity was virtually the same in all experimental groups (see Fig. 2). We found no increase in poly(U)-directed phenylalanine incorporation 30 min after CCl_4 feeding, although it has been reported for rats sacrificed 2 hr after intoxication.[4,5] These results seem to suggest that stimulation by poly(U) of phenylalanine uptake depends not on the alteration of sedimentation pattern, which accounts for the impaired incorporation directed by endogenous mRNA, but follows the complete dissociation of polysomes into particles devoid of mRNA.

Our results clearly show protection by both glutathione and propyl gallate on the deranged *in vitro* uptake of amino acids by liver microsomes of CCl_4-poisoned rats.

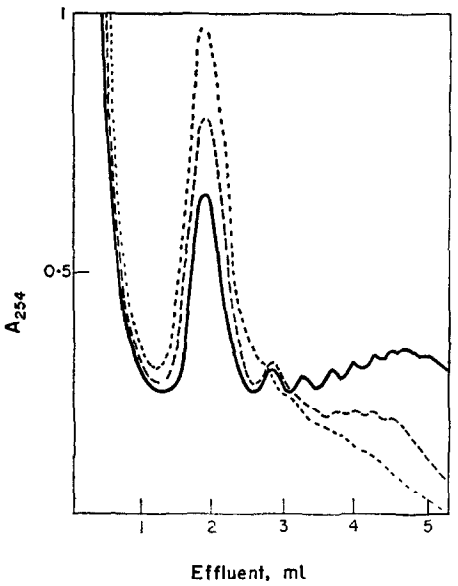

FIG. 3. Sedimentation pattern of polysomes of control rats (solid line), and of CCl_4-intoxicated rats (dotted or dashed lines). The top of the gradient is to the left. For explanation, see text.

This fact is consistent with the protective effect of these hydrosoluble antioxidants on the CCl_4-induced fatty liver.[14,19,20]

Glutathione might operate in detoxicating lipoperoxides by means of the well known GSH-peroxidase system.[6–10] In addition, glutathione and propyl gallate have been reported to react with free radicals formed on protein due to radiation,[21] hence free radical scavenging properties might also explain the radioprotection by these two compounds.[22,23] The protective effect of propyl gallate may be attributed also to inhibition of chain oxidation processes induced by radiation[24] and is still present when the gallic acid ester undergoes radiolysis[25] and becomes a protecting free radical.[26] Since the early injury induced by CCl_4 appears to be mediated at least in part by the prooxidant effect of this halogenate hydrocarbon,[27] the protective action of glutathione and propyl gallate seems to suggest that lipoperoxidation plays a role in the CCl_4-induced depression of protein synthesis. Research work is now in progress in our laboratory designed to detect and possibly estimate the inhibitory effect of lipoperoxides.

Acknowledgement—This work was aided by a grant from the Consiglio Nazionale delle Ricerche, Roma.

REFERENCES

1. K. S. RAO and R. O. RECKNAGEL, *Exp. Mol. Path.* **10**, 219 (1969).
2. K. S. RAO and R. O. RECKNAGEL, *Exp. Mol. Path.* **9**, 271 (1968).
3. E. A. SMUCKLER and E. P. BENDITT, *Biochemistry* **4**, 671 (1965).
4. J. MAGER, S. BORNSTEIN and A. HALBREICH, *Biochim. biophys. Acta* **95**, 682 (1965).
5. M. E. WEKSLER and H. V. GELBOIN, *Biochim. biophys. Acta* **145**, 185 (1967).
6. C. LITTLE and P. J. O'BRIEN, *Biochem. biophys. Res. Commun.* **31**, 145 (1968).
7. P. J. O'BRIEN and C. LITTLE, *Can. J. Biochem.* **47**, 493 (1969).
8. B. O. CHRISTOPHERSEN, *Biochem. J.* **106**, 515 (1968).
9. B. O. CHRISTOPHERSEN, *Biochim. biophys. Acta* **176**, 463 (1969).

10. R. E. PINTO and W. BARTLEY, *Biochem. J.* **115,** 449 (1969).
11. R. P. SUTTER and K. MOLDAVE, *J. biol. Chem.* **241,** 1698 (1966).
12. D. H. ALPERS, M. SOLIN and K. J. ISSELBACHER, *Molec. Pharmac.* **4,** 566 (1968).
13. E. GRAVELA and M. U. DIANZANI, *FEBS Letters* **9,** 93 (1970).
14. M. U. DIANZANI and G. UGAZIO, Commun. 2nd Intl. Symposium of Biochemical Pathology, Oxford, 13 July 1969.
15. J. STAHL, G. R. LAWFORD, B. WILLIAMS and P. N. CAMPBELL, *Biochem. J.* **109,** 155 (1968).
16. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
17. A. R. HENDERSON, *Anal. Biochem.* **27,** 315 (1969).
18. D. H. ALPERS and K. J. ISSELBACHER, *Biochim. biophys. Acta* **158,** 414 (1968).
19. G. UGAZIO and M. V. TORRIELLI, *Biochem. Pharmac.* **18,** 2271 (1969).
20. G. UGAZIO, I. GATTI, C. MAZZARINO and E. BURDINO, in preparation.
21. I. I. SAPEZHINSKII and E. G. DONTSOVA, *Biofizika* **12,** 298 (1967).
22. S. BONOTTO and M. S. NETRAWALI, *Int. J. rad. Biol.* **15,** 275 (1969).
23. E. B. BURLAKOVA, B. D. GAINTZEVA, L. B. SLEPUKHINA, N. G. KHRAPOVA and N. M. EMMANUEL, *Dokl. Akad. Nauk.* **155,** 1398 (1964).
24. A. A. GORODETSKII, V. A. BARABOI and V. P. CHERNETSKII, *Vopr. Biofiz. i Mekhanizma Deistoya Ionizir. Radiatsii*, p. 159 (1964).
25. N. M. EMMANUEL, E. B. BURLAKOVA, K. E. KRUGLIAKOVA and I. I. SEPEZHINSKII, *Izvest. Akad. Nauk.S.S.S.R.* p. 183 (1966).
26. L. KH. EIDUS, *Izvest. Akad. Nauk. S.S.S.R.* p. 383 (1966).
27. R. O. RECKNAGEL, *Pharmac. Rev.* **19,** 145 (1967).