The Reaction of Liver Proteins with a Metabolite of Carbon Tetrachloride

By C. CESSI, C. COLOMBINI and L. MAMELI

*Istituto di Patologia generale, Università di Cagliari and
Istituto di Chimica generale, Università di Padova, Italy*

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It has been suggested by Butler (1961) that the necrogenic effect of carbon tetrachloride in liver may arise from a metabolic step leading to the homolytic fission of this substance. A neutral chlorinated radical, a potential source of several noxious reactions in living systems, would be produced. Alkylation of reactive groups of proteins and peptides may occur with damage to biological activity. The fact that a product of transformation of carbon tetrachloride is the active species responsible for the toxicity is generally accepted and is supported by experimental evidence, e.g.: $^{14}\text{CO}_2$ is expired by rats treated with $^{14}\text{CCl}_4$; liver proteins become labelled in the same animals; and the necrogenic effect is relevant only in liver when the organ metabolizes the substance. These and other points have been discussed by Slater (1966), who stressed the possibility of a free radical among the metabolites of carbon tetrachloride.

The pathway from carbon tetrachloride to carbon dioxide, although not excluding homolytic fission, may involve acylating compounds that are able as well to attack the reactive group of proteins. The metabolism of carbon tetrachloride in liver and the problem of the formation of oxidized products such as formaldehyde (Heppel & Porterfield, 1948) and perhaps carbonyl chloride were investigated by Paul & Rubinstein (1963) and by Rubinstein & Kanics (1964). They obtained indirect evidence of toxic oxidized metabolites.

The investigation reported here began with the consideration of the similarity between the necrogenic effect of acylating agents such as carbonyl chloride and keten and that of carbon tetrachloride, and with the observation that liver proteins of animals administered with $^{14}\text{CCl}_4$ release $^{14}\text{CO}_2$ on acid hydrolysis, suggesting the formation of carbonyl derivatives. The behaviour of ϵ -amino groups of lysine was investigated before that of any other reactive group of peptides and proteins. Poly-L-lysine, as a model substance with accessible ϵ -amino groups only, and serum albumin were acylated by reaction with carbonyl chloride. Some properties of the carbonyl derivatives were compared with those of proteins extracted from livers of rats that had been treated with $^{14}\text{CCl}_4$.

Experimental. Poly-L-lysine hydrochloride, (Mann Research Lab. Inc., New York, N.Y., U.S.A.)

(20 mg.) was allowed to react with 10 mg. of $^{14}\text{COCl}_2$ (0.1 mc) dissolved in a small amount of tetrahydrofuran. After evaporation of the solvent and excess of reagent, the [^{14}C]carbonylpolylysine obtained was tested for resistance to acid hydrolysis. Human serum albumin (Sigma Chemical Co., St Louis, Mo., U.S.A.) (100 mg.) was treated in a similar way with 0.2 mc of $^{14}\text{COCl}_2$, and the carbonyl derivative was digested with Pronase. The peptides were fractionated on a column of Sephadex G-50 and then on a column of Dowex 1(X2) according to Braunitzer, Rudloff & Hilschmann (1963). Radioactive fractions were tested for homogeneity by thin-layer chromatography, with butanol-water-acetic acid (6:2:2, by vol.) and phenol-water (3:1 w:v) as eluents. Spots of peptides obtained from serum albumin treated with $^{14}\text{COCl}_2$ were active enough to allow radioautography in a few weeks exposure. Fractions, found to be homogeneous, were hydrolysed in hydrochloric acid and analysed for amino acid composition.

Proteins labelled with ^{14}C after administration of $^{14}\text{CCl}_4$ were obtained from rat livers. A 0.1 ml. (10 mc.) sample of $^{14}\text{CCl}_4$ suspended in 1.5 ml. of water was injected into the stomach of starving rats weighing approximately 200 g. After 16 hr. the animals were killed, the livers homogenized and the subcellular fractions separated. Proteins were prepared from each fraction essentially by the method of Siekevitz (1952), digested with Pronase and fractionated as described for the reaction product of serum albumin with $^{14}\text{COCl}_2$. The rates of loss of $^{14}\text{CO}_2$ from labelled polylysine, peptides from serum albumin treated with $^{14}\text{COCl}_2$ and peptides from liver proteins upon acid hydrolysis were tested by heating the fractions in sealed tubes at 110° with 6N-sulphuric acid for periods of time ranging from 1 to 12 hr. The $^{14}\text{CO}_2$ evolved was recovered as barium carbonate and counted.

Results and Discussion. The reaction of $^{14}\text{COCl}_2$ with polylysine and serum albumin yielded highly labelled derivatives. Although the largest proportion of radioactivity given to rats as $^{14}\text{CCl}_4$ was extracted with solvents in the course of preparation of proteins from livers, substantial incorporation was obtained with the doses employed. The activity was retained by peptides after enzymic hydrolysis and chromatography and was recovered

Fig. 1. Percentage of radioactivity recovered as $\text{Ba}^{14}\text{CO}_3$ during acid hydrolysis (6N-sulphuric acid at 110°) of $[^{14}\text{C}]$ carbonylpolylysine (●), peptides from $[^{14}\text{C}]$ carbonyl derivative of serum albumin (○), and peptides from liver proteins labelled *in vivo* by administration of $^{14}\text{CCl}_4$ (□) to rats, as function of hydrolysis time.

as carbon dioxide by acid hydrolysis. In Fig. 1 the fractions of radioactivity recovered as carbon dioxide by acid hydrolysis from $[^{14}\text{C}]$ carbonylpolylysine, from peptides of albumin treated with $^{14}\text{COCl}_2$, and from peptides of liver proteins of rats treated with $^{14}\text{CCl}_4$ are plotted against times of hydrolysis. Carbonylpolylysine proved to be less resistant to acid hydrolysis than the related homocitrulline (Stark, Stein & Moore, 1960). The rate of decomposition in acid of labelled peptides either obtained from the reaction product of serum

albumin with carbonyl chloride or from liver proteins, was comparable to that of carbonylpolylysine, with the exception of the shorter times of hydrolysis, suggesting that mainly ϵ -amino groups of lysine are involved in both reactions, with the formation of carbonyl derivatives. The formation of other, more labile carbonyl compounds may be responsible for the different behaviour of carbonylpolylysine and of carbonyl-peptides at the beginning of the hydrolysis. The analysis of amino acids of the labelled peptides always showed the presence of lysine in addition to other amino acids with reactive side chains. One can conclude that a metabolite of carbon tetrachloride in liver reacts with ϵ -amino groups of proteins, and possibly with other groups, leading to cross-linked carbonyl derivatives with impaired biological activity.

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