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Carbon tetrachloride metabolism in sheep and in *Fasciola hepatica*

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Summary

1. The excretion of carbon tetrachloride and its metabolites in bile and urine were studied.

2. Liver flukes *in vitro* metabolized carbon tetrachloride and hexachloroethane by dechlorination.

3. Carbon tetrachloride, liver lipid from rabbits which received carbon tetrachloride and a carbon tetrachloride methyl oleate complex were toxic to liver flukes *in vitro*, in the presence of sheep bile.

4. A direct fasciocidal action of carbon tetrachloride may contribute to the therapeutic effect of the drug.

Introduction

Orally administered carbon tetrachloride and hexachloroethane are effective against mature liver flukes (*Fasciola hepatica*) which reside in the bile ducts of sheep and cattle (Gibson, 1962).

Recent evidence has suggested that metabolism or activation precedes the toxic actions of carbon tetrachloride (Slater, 1966). Direct fasciocidal action could occur if the flukes themselves metabolized the drug. Carbon tetrachloride is well tolerated by newborn rats (Cameron & Karunaratne, 1936), by cockerels and by ducks (Fowler, 1970), and metabolites have not been detected in the latter.

Normally liver flukes absorb nutrients and foreign substances through the tegument or intestinal caecae (Pantelouris, 1965); some blood is utilized (Todd & Ross, 1966) and bile duct epithelial cells may be ingested (Dawes, 1963). Drugs may thus enter liver flukes *in vivo* from bile, blood or tissue cells. Alexander & Macdonald (1960) were unable to detect carbon tetrachloride in sheep bile using a microdiffusion technique sensitive to concentrations greater than 5 μg/ml (Conway, 1957) and suggested that the fasciocidal action may be due to the liver damage produced by the drug. Khalidi & Zaki (1969), using $^{14}CCl_4$, confirmed that sheep do not excrete volatile radioactivity in the bile and proposed a similar hypothesis for the mode of fasciocidal action of carbon tetrachloride.

The formation of toxic derivatives of carbon tetrachloride by liver flukes, for example, trichloromethylated lipids (Gordis, 1969), could also explain fasciocidal action. Most attention has been focused on water-soluble fasciocidal metabolites (Alexander & Macdonald, 1960; Khalidi & Zaki, 1969). In rats, water soluble radioactivity appeared as organic anions containing labelled carbon; ^{36}Cl appeared as chloride ion (Gordis, personal communication).

In order to study the fate of carbon tetrachloride in liver flukes and the action of biological fluids and extracts, particularly with regard to lipid and water soluble material, the parasites were incubated *in vitro* in several media, with and without sheep bile.

Gas liquid chromatography (GLC) was utilized as a sensitive method for detection and estimation of hexachloroethane (Fowler, 1969a), carbon tetrachloride (Fowler, 1969b) and their metabolites in extracts of liver flukes. GLC was also used to investigate excretion of carbon tetrachloride in sheep bile and urine ; with an electron capture detector, the method was suitable for estimation of fractions of a picogram of the drug (less than 1 ng/ml of extract).

Methods

Collection of sheep bile

A 3·2 mm (outer diameter) silicone rubber cannula was inserted in the bile duct of sheep 40 (Cheviot cross ; female ; 25 kg) and sheep 60 (Blackface cross ; castrated male ; 20 kg). In sheep 60 the gall-bladder and cystic duct were functionally obliterated by a 5 mm (O.D.) silicone rubber cannula introduced through the gall-bladder into the cystic duct, which enabled flushing out of the system. Bile was returned to the duodenum by a second 3·2 mm (O.D.) cannula. Bile duct and duodenal cannulae were exteriorized to the right sub-lumbar fossa and connected by a polyethylene non-return valve (Griffin S 42-630) with adaptors. This obviated blockage of bile cannulae by aspiration of duodenal contents and reduced risk of ascending biliary infection by preventing reversal of bile flow during duodenal activity. A ruminal cannula with fenestrated flange was also inserted (Alexander & Chowdhury, 1958). Bile was collected in sterile 2 litre polyethylene bags supported by a non-crushable harness on the sheep.

The operation was conducted under thiopentone sodium and cyclopropane anaesthesia ; 2 weeks' post-operative recovery were allowed before collection of bile.

Collection of rabbit bile

The contents of the gall-bladders of five rabbits were taken 6 h, 24 h and 48 h after carbon tetrachloride administration.

Collection of sheep urine

Urine was collected by the method of Warwick (1969) from castrated male sheep for determination of volume, pH, specific gravity (SG), Cl^- concentration and chlorinated hydrocarbons. The urine receiver was cooled by an ice-water bath.

Urine was collected from female sheep with a silicone rubber retention catheter (Folatex 60:8 FG) for determination of phenolsulphonephthalein (PSP) clearance after carbon tetrachloride administration.

Phenolsulphonephthalein (PSP) clearance

PSP clearance was determined 24 h, 48 h and 11 days after carbon tetrachloride administration and compared with normal clearance rates. 4 mg PSP/kg was

injected into the jugular vein of two female Cheviot cross sheep at zero time ; urine was collected for 90 min in 15 min aliquots. Each sheep received a 4 litre water load 1 h before injection of PSP. Samples were diluted with 0·5 N sodium hydroxide and optical density determined at 550 nm on an EEL spectrophotometer. The proportion of PSP excreted was determined for each 15 min period and compared with control values.

Storage of biological samples

Bile and urine were used immediately or stored at −20° C. Unfrozen bile was used in fluke incubation experiments.

Analysis of bile and urine samples from sheep

Specific gravity was determined with a hydrometer at laboratory temperature. Chloride concentration was determined by an EEL Chloridemeter and pH by a Marconi TF 1093 pH meter.

Bile and urine were extracted as described previously (Fowler, 1969a), but with heptane as extracting solvent ; extracts were examined by GLC.

Extraction of rabbit gall-bladder bile

The contents of rabbit gall-bladders were extracted as described elsewhere for rabbit tissues (Fowler, 1969b). Extracts were examined by GLC.

Gas chromatography of extracts from bile and urine

Heptane extracts of sheep bile and urine and of rabbit gall-bladder bile were examined for carbon tetrachloride and metabolites by GLC on four columns (Fowler, 1969b).

Maintenance of experimental animals and drug administration

Sheep were maintained indoors with hay and water freely available. Urine and short interval bile collections were made with animals confined to metabolism crates.

Rabbits received British Pelleted Diet and water *ad lib.*

Carbon tetrachloride was administered to rumen fistulated sheep from an all-glass syringe as a 20% (v/v) solution in olive oil, direct to the rumen. Carbon tetrachloride was administered to non-fistulated sheep and to rabbits by stomach tube as a 20% solution in olive oil. Sheep 40 and 60 received 3 ml carbon tetrachloride (0·12 and 0·15 ml/kg respectively) and rabbits received 1 ml/kg.

Liver fluke in vitro *studies*

Viable liver flukes obtained from sheep bile ducts within 1 h of slaughter were washed with Hedon-Fleig solution (Gatenby, 1937) at room temperature. Hedon-Fleig solution also contained glucose (0·005 M), procaine penicillin (5×10^5 units/l.) and streptomycin sulphate (0·6 g/l.). Entire, motile flukes were incubated one per tube at 37° C with emulsions of drugs or various media. Flukes were examined frequently during incubation and always 3, 5, 10, 21 and 26 h after the start of

incubation for signs of movement. Non-motile flukes were stimulated with a 15 V d.c. current at 60 Hz for 3 s to test their ability to respond.

Inactivated flukes were obtained by heating viable flukes for 5 min in saline (0·15 M) at 100° C.

Preparation of emulsions

A primary emulsion was prepared in a pestle-and-mortar with olive oil, acacia and water in the ratio 4:2:1. Drugs were dissolved in olive oil before emulsification. Primary emulsions were diluted with Hedon-Fleig solution with shaking and contained droplets 1·4–5·0 μm in diameter (95%) when measured by microscope stage micrometer.

Methyl oleate/carbon tetrachloride addition product

The addition product ("trichloromethylated oleate") was prepared as described by Gordis (1969) and was purged with nitrogen for 18 h at 100° C to remove volatile contaminants.

Preparation of rabbit liver extracts

Of four New Zealand White rabbits (1·5–1·8 kg), two received carbon tetrachloride by stomach tube (2 ml/kg). Two hours later, control and treated rabbits were stunned, bled, the livers removed and ground with acid-washed silversand in glass pestles and mortars under nitrogen. The macerated livers were extracted three times with ether (total 300 ml), the extracts combined and the ether evaporated off at room temperature under a reduced pressure of nitrogen.

The aqueous mass was centrifuged for 30 min at RCF 1,600 to remove tissue debris and the supernatant was stored frozen at −20° C. 25 ml of aqueous liver extract was equivalent to about 12·5 g ether extracted liver tissue. The ether extracted material was also stored at −20° C under nitrogen to protect unsaturated fatty acids from oxidation.

Gas chromatography of fluke extracts

After incubation, liver flukes were blotted dry, weighed, homogenized, extracted with heptane and examined by GLC as described for rabbit tissue (Fowler, 1969b). In experiments with hexachloroethane emulsion, fluke homogenates were extracted with hexane before GLC analysis (Fowler, 1969a).

Reagents

Analar grade reagents were used where available. Hexane fraction of petroleum and *n*-heptane (British Drug Houses), carbon tetrachloride and hexachloroethane were purified as described elsewhere (Fowler, 1969a, b).

Olive oil and powdered acacia were B.P. grade.

Methyl oleate was oleic acid methyl ester (Sigma).

Results

Gas chromatography showed that carbon tetrachloride, chloroform and hexachloroethane were present in gall-bladder bile of rabbits which had received carbon

tetrachloride (Table 1). Carbon tetrachloride was also detected in the bile of sheep within 3 min of intra-ruminal administration. The highest biliary concentration of the drug (4–5 μg/ml) occurred 1–3 h after administration and fell to less than 1 μg/ml 6 h after dosage.

Chloroform was also excreted in the bile of sheep which received carbon tetrachloride and traces of carbon tetrachloride and chloroform were found in the urine of sheep which received the drug (Table 2).

The pH of sheep bile increased after carbon tetrachloride administration and returned to normal 6–7 days later; biliary volume decreased sharply and remained depressed for 12–14 days. pH of sheep urine did not change markedly although an initial, transient diuresis was observed; no change in specific gravity of urine was seen but that of bile increased after administration of the drug. The concentration of chloride ion decreased in bile and in urine.

Phenolsulphonephthalein clearance was unaffected by administration of carbon tetrachloride.

In vitro *studies of liver fluke motility*

Liver flukes retained motility for 2–3 days *in vitro* when incubated in Hedon-Fleig solution (Table 3). Other media were less suitable for maintenance of *F. hepatica in vitro* (Table 3). Emulsions of liver lipid from carbon tetrachloride treated rabbits, of carbon tetrachloride and of its addition product with methyl oleate were especially toxic in the presence of sheep bile.

Gas chromatography of liver fluke extracts

Neither carbon tetrachloride nor its metabolites were detected in normal flukes. Flukes incubated in carbon tetrachloride emulsions took up the drug. Chloroform and hexachloroethane were also detected in the flukes when 25% sheep bile was added to carbon tetrachloride emulsions (Table 4).

TABLE 1. *Volatile chlorinated constituents of gall-bladder bile from rabbits which received carbon tetrachloride (1 ml/kg)*

Time after dose	CCl_4 μg/g ±S.D.	$CHCl_3$ μg/g±S.D.	$Cl_3C.CCl_3$ ng/g±S.D.
6 h	37±7	0·50±0·12	Trace
24 h	7·8±1·5	0·14±0·02	5·5±1·8
48 h	1·1±1·3	0·45±0·21	Trace

TABLE 2. *Comparison of total amounts of carbon tetrachloride and chloroform in sheep bile and urine (μg) following administration of carbon tetrachloride*

Dose	Sheep 37 0·1 ml/kg Urine		Sheep 38 0·12 ml/kg Urine		Sheep 40 0·12 ml/kg Bile		Sheep 60 0·15 ml/kg Bile	
	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$
0–6 h	—	—	—	—	398	—	438	—
0–1 day	19·2	3·7	1·2	6·6	433	241	543	210
1–2 days	5·9	2·0	1·0	3·3	7	122	9	126
2–3 days	4·6	1·8	0·7	2·2	6	95	8	120
3–4 days	1·3	0·8	0·7	2·0	5	50	2	20
4–5 days	0·6	0·2	0·5	0·2	5	Nil	1	Nil
5–6 days	Trace	Trace	Trace	Trace	Trace	Nil	Nil	Nil
6–7 days	Trace	Trace	Trace	Trace	Trace	—	Nil	Nil

Viable and inactivated flukes incubated in hexachloroethane emulsions took up the drug; viable flukes also contained pentachloroethane and tetrachloroethylene but did not lose motility during 4 h incubation. Inactivated flukes did not contain metabolites of hexachloroethane (Table 5).

Discussion

Gas chromatography was found to be a most sensitive method for detection of carbon tetrachloride in bile. Conventional micro-diffusion techniques (Conway, 1957) are not sufficiently sensitive, have led to misleading results and failure to identify carbon tetrachloride in the bile of sheep which received therapeutic doses of the drug (Alexander & Macdonald, 1960). Furthermore, a technique usine ^{14}C labelled drug (Khalidi & Zaki, 1969) failed to demonstrate its excretion in the bile; it is possible that loss of volatile biliary constituents or severe quenching effects may

TABLE 3. *Motility of liver flukes incubated at 37° C*

Composition of medium	No. of flukes	Sheep bile	50% lost motility in:
Hedon-Fleig solution	20	Nil	74 h
Normal sheep bile	30	25%	12 h
Bile 0–24 h after CCl_4	10	25%	26 h
Bile 24–48 h after CCl_4	10	25%	15 h
Control aqueous rabbit liver extract 25%	10	Nil	11 h
CCl_4 treated aqueous rabbit liver extract 25%	10	Nil	26 h
Control rabbit liver lipid 1%	16	25%	10 h
CCl_4 treated rabbit liver lipid 1%	16	25%	2 h
Olive oil emulsion	10	25%	28 h
CCl_4 in olive oil emulsion	33	25%	1 h
Hedon-Fleig solution	12	Nil	86 h
CCl_4 in olive oil emulsion	11	Nil	63 h
Oleic acid (methyl ester) 1%	8	Nil	26 h
Hedon-Fleig solution	28	Nil	63 h
CCl_4 in olive oil emulsion	11	Nil	46 h
Oleic acid (methyl ester) ½%	14	25%	15 h
Oleic acid (methyl ester) CCl_4 treated ½%	20	25%	7 h
Hedon-Fleig solution	10	Nil	76 h
Olive oil emulsion	10	25%	32 h
CCl_4 in olive oil emulsion	12	25%	1 h
Oleic acid (methyl ester) CCl_4 treated 1%	20	25%	1 h

Media emulsified with Hedon-Fleig solution.

TABLE 4. *Concentration of carbon tetrachloride, chloroform and hexachloroethane (μg/g±S.D.) in fluke tissues after incubation in carbon tetrachloride emulsion*

CCl_4 in medium (μg/ml)	Flukes: Weight (mg±S.D)	Flukes: No.	Tissue extracts: CCl_4	Tissue extracts: $CHCl_3$	Tissue extracts: $Cl_3C.CCl_3$
50± 5	102±12	17	76± 8	0·38±0·04	0·19±0·01
93±14	161±22	10	141± 5	0·57±0·06	0·22±0·02
126±19	160±15	18	223±71	1·91±0·21	0·38±0·06

TABLE 5. *Concentrations of hexachloroethane, pentachloroethane amd tetrachloroethylene (μg/g ±S.D.) in fluke tissues after incubation in hexachloroethane emulsion*

C_2Cl_6 in medium (μg/ml)	Flukes: Weight (mg±S.D)	Flukes: No.	Tissue extracts: C_2Cl_6	Tissue extracts: C_2HCl_5	Tissue extracts: C_2Cl_4
14·2±0·3	290±58	19	8·51±0·40	0·19±0·06	0·55±0·14
16·0±2·0	263±82	10*	13·8±0·90	Nil	Nil
21·8±0·3	257±57	16	17·49±2·45	0·16±0·04	0·64±0·17

*Flukes inactivated by boiling.

have occurred (Turner, 1967) and almost certainly a different breed of sheep was involved. A method involving heating of bile to drive off labelled carbon tetrachloride (Khalidi & Zaki, 1969) may introduce a further inaccuracy: on repeating this method (with $^{13}CCl_4$) it was found that 29–64% of carbon tetrachloride (determined by gas chromatography) was not displaced.

The toxicity of carbon tetrachloride to liver flukes *in vitro* is not in agreement with results of some other workers. A toxic action was observed in the presence of bile; bile may have stabilized the drug emulsions, changed the distribution of toxic substances within the flukes or reacted with carbon tetrachloride to form a toxic product. Although the mechanism is not known, exposure of parasites to emulsions rather than solutions of drugs may increase the activity of the drugs (Baldwin, 1943) and may more closely resemble the *in vivo* situation. Emulsions used were of relatively small droplet size and may be likened to large micelles such as occur in mammalian bile (Masoro, 1968).

Several mechanisms may be postulated for a fasciocidal action of carbon tetrachloride *in vivo*:

(1) *An indirect action through release of products of liver damage.* The anthelmintic action of carbon tetrachloride on liver flukes may be due to liver damage produced by the drug and not through a direct action on the fluke in the bile duct (Alexander & Macdonald, 1960). This hypothesis was further developed by Khalidi & Zaki (1969), who suggested that damaged liver cells may release products lethal to liver flukes. Such products might occur in the aqueous or in the lipid fraction. In support of this hypothesis, ether extractable material from carbon tetrachloride treated rabbits' livers was found to be toxic to liver flukes *in vitro*.

Toxic compounds could arise from the interaction of carbon tetrachloride with liver lipids. Trichloromethylated oleate (Gordis, 1969) was also toxic to liver flukes *in vitro* and may resemble the lipids excreted after carbon tetrachloride administration. Bile from sheep which had received carbon tetrachloride, however, was not toxic to liver flukes in the present experiments. It is possible that the toxic agent is volatile or unstable and was lost during freezing, storage or thawing of bile. Furthermore, alteration of physical characteristics of bile, for example, micelle structure, could alter toxic properties of the bile. *In vitro* results may not, perhaps, be relevant to the *in vivo* situation.

(2) *An indirect action through changing the environment.* The reduction in bile flow after carbon tetrachloride administration may lower amount of substrates available to flukes or may allow excretory products to reach toxic levels.

Moreover, the pH of the bile was seen to rise in these experiments and may have contributed to a fasciocidal action. The liver fluke is not a strict anaerobe (Thorsell, 1963) and a high P_{CO_2} due to biliary stasis may prove toxic. Biliary flow rates found in the non-medicated sheep were similar to those described by Mortimer & Stanbridge (1969) with enterohepatic circulation maintained, but much greater than those reported by Khalidi & Zaki (1969).

(3) *A direct fasciocidal action of carbon tetrachloride.* The liver fluke *in vitro* was able to metabolize carbon tetrachloride. Hexachloroethane, which may arise by dimerization of free trichloromethyl radicals (Fowler, 1969b), was detected in extracts of liver flukes and its presence may provide evidence of activation (Slater,

1966). Activation of the drug within fluke tissues may provide a direct fasciocidal mechanism.

Hexachloroethane, although metabolized, was not markedly toxic to liver flukes *in vitro* and it seems unlikely that the fasciocidal actions of carbon tetrachloride can be attributed to synthesis of this substance by the flukes.

Carbon tetrachloride is toxic to flukes in the presence of liver slices (Kondos & McClymont, 1965) and it has now been established that the drug is present in bile for at least 6 h following dosage. It seems likely, therefore, that adult liver flukes have direct access to carbon tetrachloride, although in much lower concentrations than those reported active *in vitro*. Since mature liver flukes *in vivo* ingest blood and tissue cells (Todd & Ross, 1966 ; Dawes, 1963) these may provide a further source of carbon tetrachloride.

Immature flukes are resistant during the 4–6 post-invasive weeks in sheep (Boray & Happich, 1968), during which period they are undergoing maturation in the liver tissues. If metabolism of carbon tetrachloride contributes to its fasciocidal action such flukes may have a reduced capacity to metabolize or activate the drug ; they are deficient in certain enzymes. Glutamate dehydrogenase and alkaline phosphatase, which are present in mature liver flukes, are less active in immature flukes (Thorpe, 1968) and may reflect a similar situation in drug metabolizing enzymes.

Direct and indirect mechanisms for the fasciocidal action of carbon tetrachloride are not mutually exclusive and both may play a part in the therapeutic action of the drug.

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REFERENCES

ALEXANDER, F. & CHOWDHURY, A. K. (1958). Digestion in the rabbit's stomach. *Br. J. Nutr.*, **12**, 65–73.

ALEXANDER, F. & MACDONALD, D. C. (1960). The action of carbon tetrachloride on the sheep's liver. *Q. J. exp. Physiol.*, **45**, 12–17.

BALDWIN, E. (1943). Chemotherapeutic investigation of anthelmintic potency. *Parasitology*, **35**, 89–111.

BORAY, J. C. & HAPPICH, F. A. (1968). Standardised chemotherapeutical tests for immature and mature *Fasciola hepatica* infections in sheep. *Aust. vet. J.*, **44**, 72–78.

CAMERON, G. R. & KARUNARATNE, W. A. E. (1936). Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. path. Bact.*, **42**, 1–21.

CONWAY, E. J. (1957). *Microdiffusion Analysis*, 4th ed. London: Lockwood.

DAWES, B. (1963). Hyperplasia of the bile duct in fascioliasis and its relation to the problem of nutrition in the liver fluke. *Parasitology*, **53**, 123–133.

FOWLER, J. S. L. (1969a). Some hepatotoxic actions of hexachloroethane and its metabolites in sheep. *Br. J. Pharmac.*, **35**, 530–542.

FOWLER, J. S. L. (1969b). Carbon tetrachloride metabolism in the rabbit. *Br. J. Pharmac.*, **37**, 733–737.

FOWLER, J. S. L. (1970). Chlorinated hydrocarbon toxicity in the fowl and duck. *J. comp. Path.*, in the Press.

GATENBY, J. B. (1937). *Biological Laboratory Technique*. London: Churchill.

GIBSON, T. E. (1962). *Veterinary Anthelmintic Medication*, 1st ed., Technical communication No. 33. Farnham Royal, Buckinghamshire: Commonwealth Bureaux of Helminthology, Commonwealth Agricultural Bureaux.

GORDIS, E. (1969). Lipid metabolites of carbon tetrachloride. *J. clin. Invest.*, **48**, 203–209.

KHALIDI, A. & ZAKI, S. A. (1969). The mode of action of carbon tetrachloride on *Fasciola hepatica*. *Br. J. Pharmac.*, **36**, 253–256.

KONDOS, A. C. & MCCLYMONT, G. L. (1965). Indirect anthelmintic action of carbon tetrachloride against *Fasciola hepatica*. *Nature, Lond.*, **206**, 846–847.

MASORO, E. J. (1968). *Physiological Chemistry of Lipids in Mammals*. Philadelphia: Saunders.

MORTIMER, P. H. & STANBRIDGE, T. A. (1969). Changes in biliary secretion following sporidesmin poisoning in sheep. *J. comp. Path.*, **79**, 267–275.

PANTELOURIS, E. M. (1965). *The Common Liver Fluke*, 1st ed. Oxford: Pergamon.

SLATER, T. F. (1966). Necrogenic action of carbon tetrachloride in the rat. *Nature., Lond.*, **209**, 36–40.

THORPE, E. (1968). Comparative enzyme histochemistry of immature and mature stages of *Fasciola hepatica*. *Exp. Parasit.*, **22**, 150–159.

THORSELL, W. (1963). Biochemical studies on the liver fluke. *Acta chem. scand.*, **17**, 884.

TODD, J. R. & ROSS, J. G. (1966). Origin of haemoglobin in the caecal contents of *Fasciola hepatica*. *Exp. Parasit.*, **19**, 151–154.

TURNER, J. C. (1967). Sample Preparation for Liquid Scintillation Counting. Review 6. Amersham, Buckinghamshire: The Radiochemical Centre.

WARWICK, I. S. (1969). Urine collection apparatus for male sheep. *J. Inst. Animal Technicians*, **20**, 104–106.

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