**Zeitschrift:** Acta Tropica
**Herausgeber:** Schweizerisches Tropeninstitut (Basel)
**Band:** 37 (1980)
**Heft:** 4

**Artikel:** "Opisthorchis viverrini" : liver changes in golden hamsters maintained on high and low protein diets
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**DOI:** https://doi.org/10.5169/seals-312670

Acta Tropica 37, 337–350 (1980)

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# *Opisthorchis viverrini:* liver changes in golden hamsters maintained on high and low protein diets

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## Summary

Two groups of hamsters maintained on either high (25.6%) or low (5.3%) content protein diets were infected with 50 *Opisthorchis viverrini* metacercariae by intragastric inoculation. Three animals from each group were sacrificed at 14-day intervals over a 32-week period. Two groups of non-infected control animals maintained on identical diets were killed at similar intervals.

Histological examination revealed qualitatively similar pathological responses to the parasite in both diet groups, but overall the low protein diet group had the more severe lesions. Two weeks after infection, second order bile ducts showed epithelial focal necrosis, reactive hyperplasia and folding of the bile duct epithelium with some periductal fibrosis. Periductal inflammatory cells were predominantly eosinophils and lymphocytes at this time, changing after six weeks to predominantly lymphoblast and plasma cell infiltrates.

Central bile ducts showed maximal concentric fibrosis at 12 weeks and this was considerably more pronounced in high protein fed animals. The small peripheral bile ductules proliferated and by four weeks post-infection, adjacent portal tracts appeared linked together, until at eight weeks some of the livers were nodular. The degree of bile ductule proliferation was markedly more pronounced in the low protein fed animals such that by 12 weeks parts of the peripheral liver substance was obliterated by proliferating ductules. No tumours or evidence of premalignant lesions were detected in livers from any of the infected animals, but it is likely that the infection period was rather too short for malignant transformation to ensue.

The possible pathogenetic mechanisms operating in this animal model of opisthorchiasis are discussed with particular reference to the disease in man.

*Key words: Opisthorchis viverrini;* hamster; protein deficiency; liver lesions; granulomas; bile duct tumours.

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## Introduction

Parasitisation by the liver fluke *Opisthorchis viverrini* is endemic among the human population of north-east Thailand (Harinasuta and Vajrasthira, 1960). Infection is acquired as a direct consequence of eating raw or undercooked fish containing encysted metacercariae of the parasite, an eating custom practiced for many centuries in this part of the world (Sornmani et al., 1973). The metacercariae upon entering the duodenum excyst and the juvenile fluke that is liberated migrates through the papilla of Vater into the common bile duct and thence into the intrahepatic bile ducts where they mature and may remain for many years (Viranuvati, 1972). Egg production commences between 3–4 weeks, and eggs pass into the duodenum and are voided with the faeces.

There is ample epidemiological and histopathological evidence strongly implicating both *O. viverrini* and *C. sinensis* as aetiological factors in the development of bile duct carcinoma in man (Hou, 1956; Purtilo, 1976; Sonakul et al., 1978). Bhamarapravati et al. (1978) have investigated experimental *O. viverrini* infections in hamsters, but failed to demonstrate tumours in animals infected for up to 154 days. However, Thamavit et al. (1978) demonstrated that hamsters fed the hepatocarcinogen, dimethylnitrosamine invariably developed bile duct carcinomas after 18 weeks whilst uninfected controls fed carcinogen only did not. Thus, it has been suggested that the development of bile duct carcinoma in the *O. viverrini* infected host is probably a multifactoral process, the presence of the parasite in the bile ducts possibly enhancing the carcinogenic activity of environmental or dietary factors. Alternatively, the parasite or its products per se may provide the only carcinogenic stimulus.

Socioeconomic studies in north-east Thailand suggest that nutrition among the indigenous population is poor, particularly with respect to protein foodstuffs (Migasena, 1972). This prompted us to investigate an experimental *O. viverrini* infection in animals maintained on low protein diets. In the present study we describe the histopathological response of hamsters at various times after infection with a standard number of metacercariae and maintained on diets of low or high protein content over a total infection period of 32 weeks.

### Materials and methods

*Hamsters.* Outbred golden Syrian hamsters, 6–12 weeks of age at commencement of the experiment were obtained from the Animal House of Chulalongkorn University, Bangkok. Animals were housed six to a cage on wood shavings and fed water and a standard pellet diet (Gold Coin Mills, Singapore) ad libitum unless otherwise stated.

*Diets.* A low protein diet providing crude protein at a 5.3% level was formulated according to the data shown in Table 1. Animals were fed ad libitum on this diet two weeks prior to receiving metacercariae and thereafter for the duration of the experiment. The standard pellet diet was also fed to animals ad libitum and provided crude protein at a 25.6% level.

*Metacercariae and experimental infections.* Cyprinoid fish infected with *O. viverrini* metacercariae were collected from paddy fields surrounding a small village in Nakorn Nayok Province, Central Thailand, and transported to the laboratory in Bangkok on ice. Metacercariae were identi-

Table 1. Compositions of the high and low protein hamster diets

| Low protein diet (5.3%) | | High protein diet* (25.6%) | |
|---|---|---|---|
| Ingredient | % of diet[1] | Ingredient | % of diet |
| Rice flour | 68 | Alfalfa meal | 2.0 |
| Potato starch | 22 | Fish meal | 7.0 |
| Corn starch | 8 | Maize meal | 10.0 |
| Vitamin mix[2] | 1 | Meat/bone meal | 2.8 |
| Mineral mix[3] | 1 | Milk powder | 6.0 |
| | | Palm oil | 0.5 |
| | | Soyabean meal | 23.5 |
| | | Tapioca meal | 3.0 |
| | | Wheat meal | 36.1 |
| | | Wheat pollard | 6.9 |
| | | Mineral mix[4] | 1.2 |
| | | Vitamin mix[5] | 1.0 |

[1] One kg quantities of the diet mix were mixed with 20 ml corn oil and one litre of water and baked at 80° C to give biscuit.

[2] Vitamin mix providing per kg of diet; Vitamin A 22,000 IU; Vitamin $D_2$ 500 IU; Vitamin $D_3$ 1000 IU; Vitamin E 20 mg; Vitamin $B_2$ 54 mg; Vitamin K 2.0 mg; Vitamin $B_6$ 120 mg; Vitamin $B_1$ 2.0 mg; Vitamin $B_{12}$ 30 $\mu$g; Choline chloride 10 g.

[3] Mineral mix providing per kg diet: iron 15 mg; Magnesium 50 mg; Zinc 50 mg; Manganese 2 mg; Copper 10 mg; Cobalt 6 mg.

[4] Mineral mix providing per kg diet; Cobalt 6.3 mg; Copper 21.7 mg; Manganese 1.9 mg; iodine 22.9 mg; Sulphur 30 mg; Iron 93.6 mg; Magnesium 66.8 mg; Zinc 50.0 mg.

[5] Vitamin mix providing per kg diet; Vitamin A 20,000 IU; Vitamin $B_{12}$ 50 $\mu$g; Vitamin $D_3$ 2000 IU; Vitamin K 1 mg; Calcium-DL-Pantothenate 100 mg; Niacin 100 mg; Riboflavin 15 mg; Vitamin E 30 mg; Ethoxyquin 125 mg; Inositol 1,500 mg; Thiamine 30 mg; Pyridoxine 7.7 mg.

* Commercial pellet diet manufactured by Gold Coin Ltd., Singapore.

fied according to the criteria of Harinasuta and Vajrasthira (1960) and carefully disected from the fish flesh under a disecting microscope. Hamsters were infected with 50 active metacercariae in 1–3 ml saline by intragastric inoculation, great care being taken to ensure that all metacercariae were flushed from the syringe and gastric tube.

*Experimental design.* Animals from two groups of 48 hamsters, one group maintained on the standard pellet diet (high protein) and the other on the low protein diet each received 50 metacercariae. A group of 48 non-infected animals maintained on the low protein diet, and a group of 27 animals on the standard pellet diet served as controls. Animals were weighed weekly in groups of six or three, and the mean weight of animals within each group was calculated.

Three animals from each of the infected groups and the non-infected control group fed the low protein diet were sacrificed at 14-day intervals up to 32 weeks post-infection. Ten animals from the control group maintained on the standard pellet diet were sacrificed at ten weeks and the remaining 17 animals at 32 weeks.

The liver and extrahepatic biliary system were removed from each animal upon sacrifice and fixed in sodium acetate buffered 10% formalin. Three portions of tissue from the right, middle and left liver lobes were taken for processing, embedded in wax, sections cut at 5 $\mu$ and stained with haematoxylin and eosin and special stains as required.

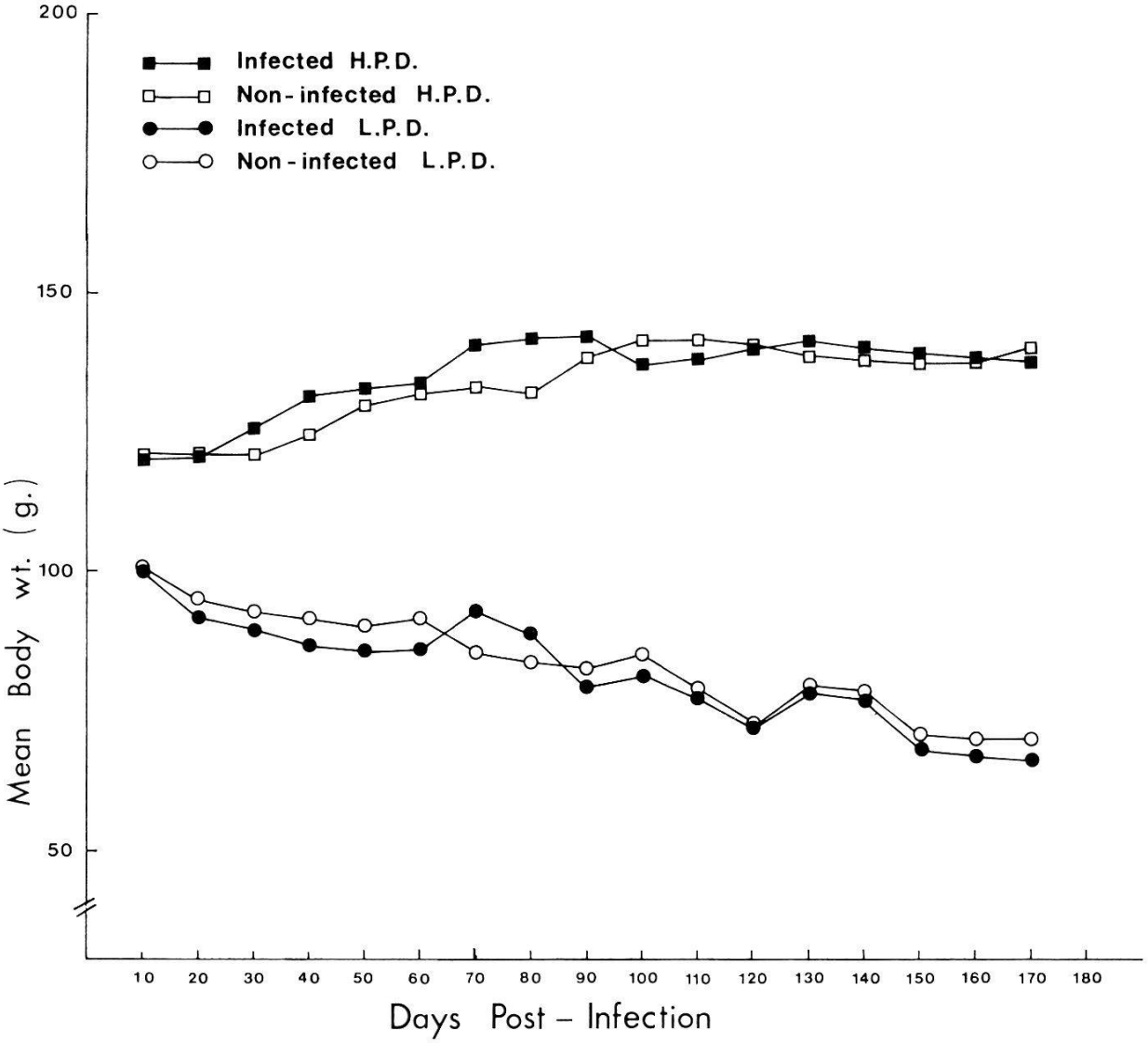


Fig. 1. Growth curves for infected and non-infected hamsters fed either the high or low protein diets. H.P.D. = high protein diet; L.P.D. = low protein diet.

## Results

The growth curves for infected and control animals fed either the low or high protein diets are shown in Fig. 1. Animals maintained on the high protein diet made significantly greater weight gains than low protein fed animals, though there was no substantial difference in weight gains or losses between infected and control animals within the same diet group.

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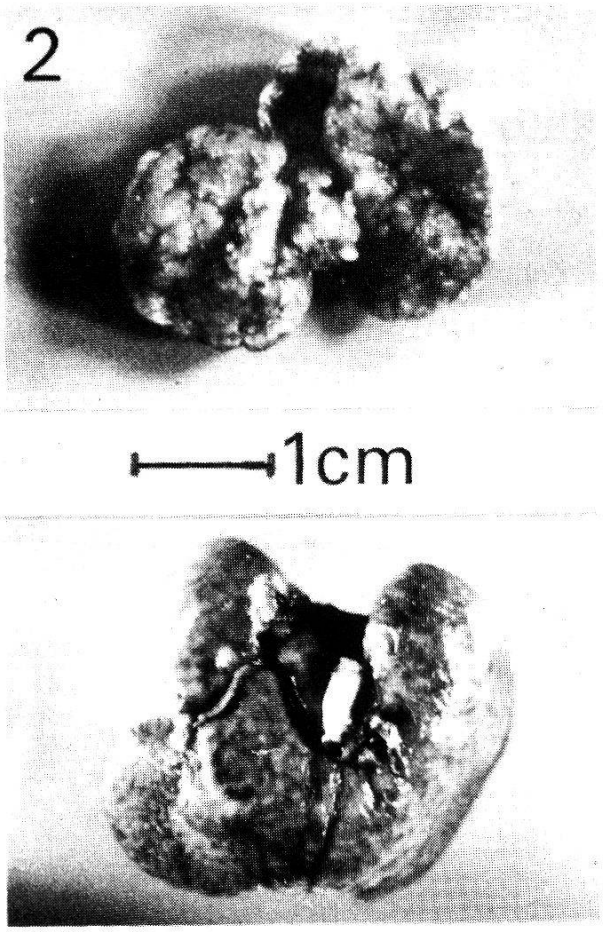
Figs. 2–11. H.P.D. = high protein diet; L.P.D. = low protein diet.

Fig. 2. Livers 10 weeks post-infection, showing granularity in the H.P.D. fed hamster (bottom) and nodularity in the L.P.D. fed hamster (top).

Fig. 3. H.P.D. hamster liver 2 weeks post-infection: the growing fluke is causing bile duct epithelial necrosis and hyperplasia (H & E, 220×).

Fig. 4. H.P.D. hamster liver 4 weeks post-infection: the bile duct epithelium is hyperplastic and polypoid (H & E, 88×).

Fig. 5. L.P.D. hamster liver 10 weeks post-infection showing lymphoid aggregates and peripheral nodularity due to ductular proliferation (H & E, 25×).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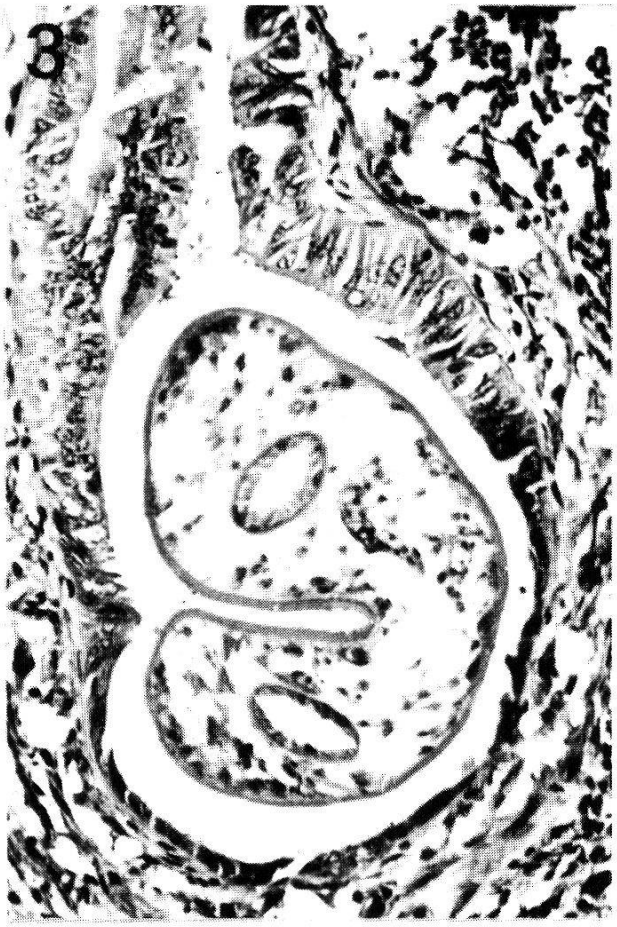
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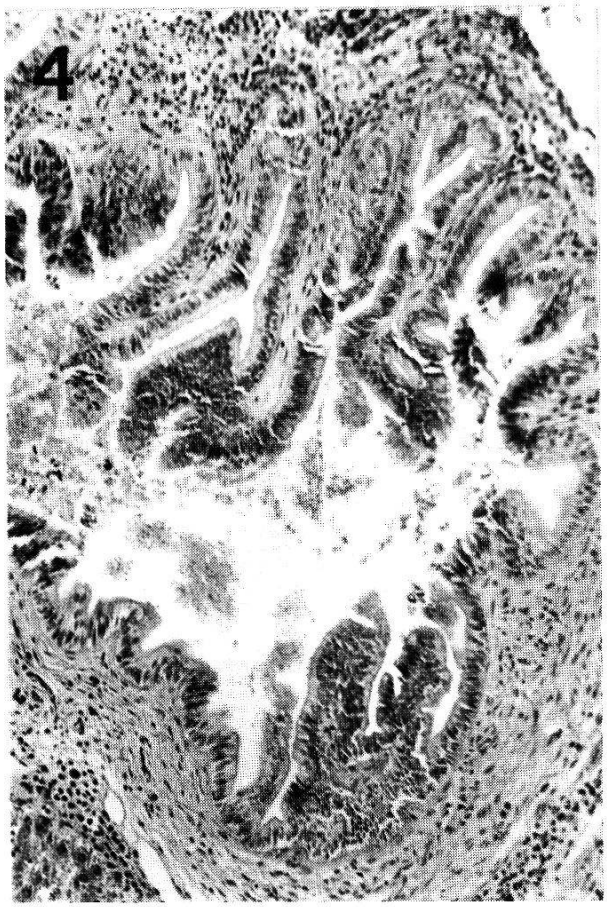
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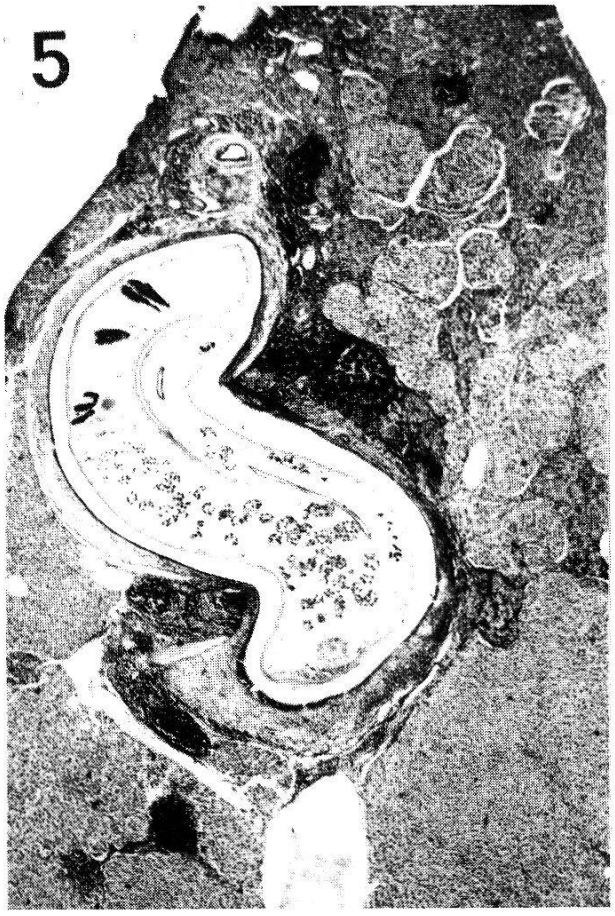
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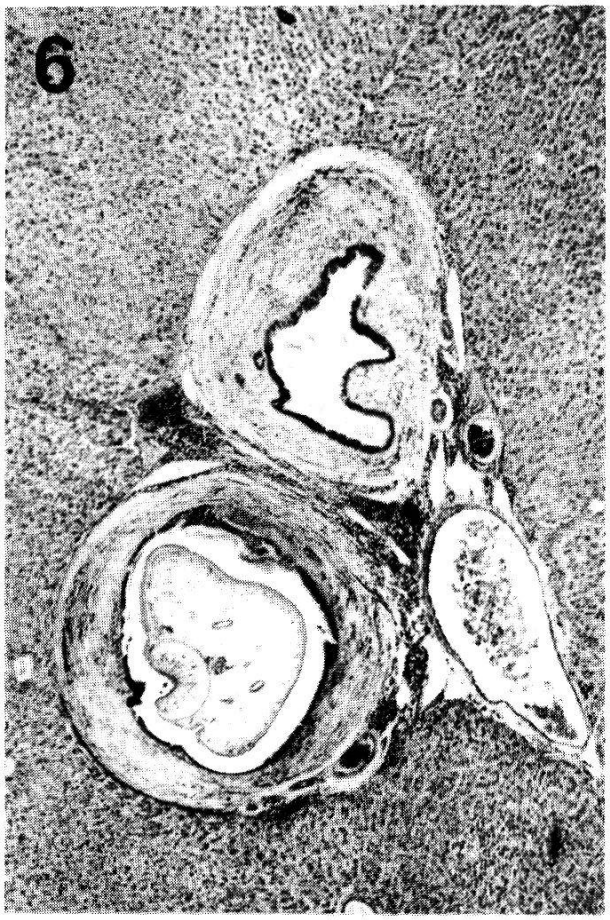
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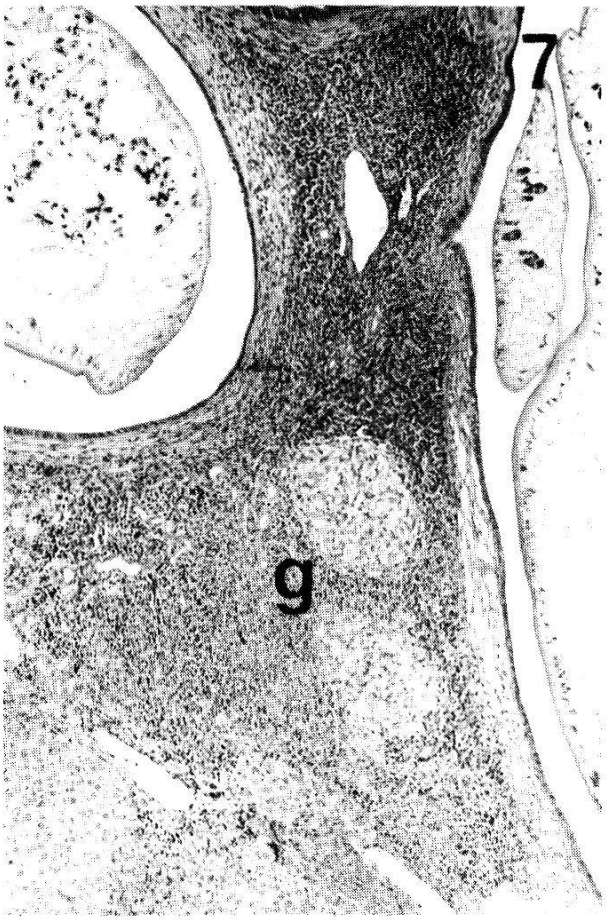
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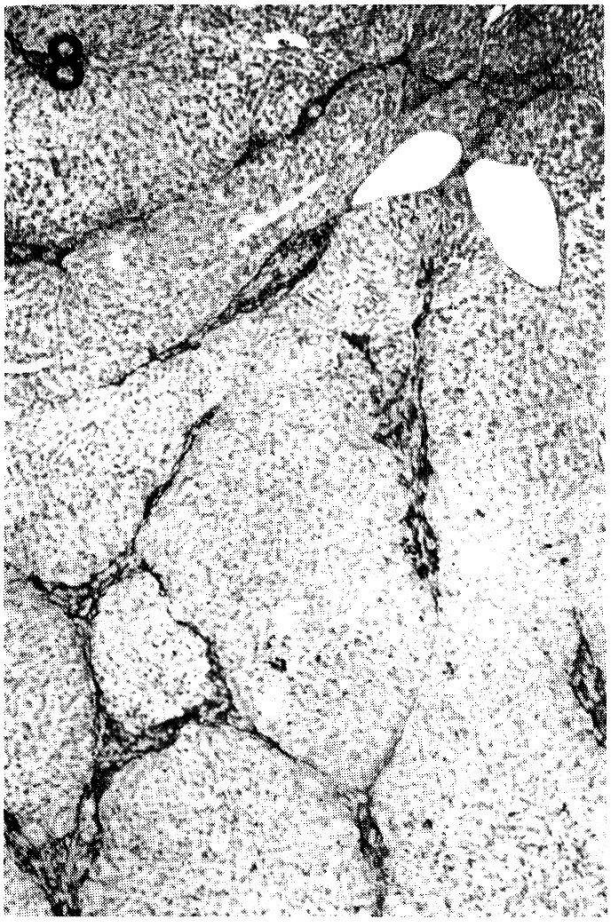
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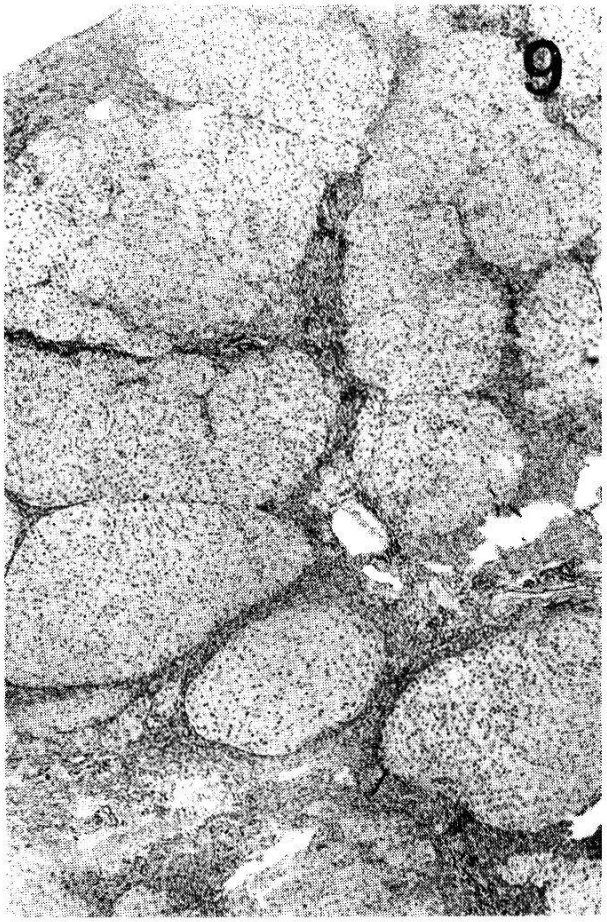
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*Pathology*

*Macroscopic features.* The livers of non-infected animals were macroscopically normal. Infected animals in both diet groups showed after four weeks of infection, focal granularity of the liver surface and prominence of the lobules. In the low protein group, this proceeded to a marked nodularity in most animals after eight weeks (Fig. 2). The cut surfaces of the liver revealed worms mainly in the region of the porta hepatis in all but three hamsters in each group.

*Microscopic features.* Non infected hamsters on the low protein diet showed only a focal mild hepatitis in a few cases. In contrast, many low protein fed control animals had liver cell atrophy with widened sinusoids and smaller hepatocytes with vacuolation and less ergastoplasm. Focal hepatocyte necrosis was also more evident in low protein fed control animals, and over the total 32 weeks of the experiment there was increasing deposition of lipofuscin and haemosiderin pigment in Kupffer cells and portal tract macrophages. Two hamsters in this group also developed periportal amyloid. None of the control animals showed bile duct proliferation or nodularity.

All infected animals demonstrated qualitatively similar reactions to the parasite but overall the low protein group had the more severe lesions. In both diet groups by two weeks, young worms without eggs were growing in the second order bile ducts near the porta hepatis, with resulting focal necrosis of the mucosa (Fig. 3), reactive hyperplasia and folding of the distended bile duct epithelium. Even at this early stage, periductal fibroblasts and thickening of the portal tract connective tissue were evident. The surrounding inflammatory reaction was predominantly eosinophilic and lymphocytic, and a similar reaction extended outward along the length of the peripheral bile ductules.

By four weeks post-infection, egg production has commenced. Adult worms, often in pairs, were distending the bile ducts, and some were found in the common bile duct and gall bladder, where they evoked no reaction. The bile duct epithelial reaction continued with regenerative mitoses and pseudo-stratification; many animals showed focal polypoid hyperplasia (Fig. 4) and goblet cell metaplasia. However, no animal in either diet group had any significant duct epithelial atypia or glandular downgrowths into the periductal connective tissues.

Around the central bile ducts there was progressive, mainly concentric fibrosis, which reached a maximum thickness by twelve weeks post-infection and persisted thereafter (Fig. 6). A consistent finding throughout the ex-

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Fig. 6. H.P.D. hamster liver 10 weeks post-infection with thick fibrous sleeve around second order bile ducts (H & E, 35×).

Fig. 7. L.P.D. hamster liver with little periductal fibrosis. Two granulomas (g) can be seen outside the area of fibrosis (H & E, 60×).

Fig. 8. L.P.D. hamster liver 4 weeks post-infection showing linkage of adjacent portal tracts (Reticulin, 35×).

Fig. 9. L.P.D. hamster liver 8 weeks post-infection with a "pseudo-cirrhosis" (H & E, 40×).

periment was the greater amount of fibrosis laid down about the second order bile ducts in the high protein fed animals compared with the low protein fed group (Figs. 6 and 7). Eggs released by adult worms were found in portal tract tissues from four weeks post-infection, presumably having reached it through breaches in the epithelium, and a few eggs were seen to have passed up the ductules against the bile flow to the peripheral portal tracts. At four weeks they elicited a simple macrophage granuloma, but after six weeks the granulomas were epitheloid cell in type, becoming confluent around one or more eggs, and they had no associated eosinophils. Central necrosis and cholesterol clefts occurred after eight weeks, and in a few animals, granulomas could be seen protruding through ulcerated epithelium into the bile duct lumen. However, most granulomas were situated outside the sleeve of periductal fibrosis (Fig. 7), and in time they hyalinized and fused with the existing fibrosis. Many of the eggs apparently did not evoke granulomatous responses but were phagocytosed into giant cells were they calcified and appeared to form Schaumann bodies.

After six weeks of infection, the periductal and periductular inflammatory reactions in both groups were less eosinophilic and lymphoblasts, plasma cells and lymph follicles with active germinal centres became more prominent (Fig. 5). Mast cells were moderately increased by infection but there was no change in mast cell numbers with diet or duration of infection. Mast cells did not appear to be degranulating at any stage of the experiment and their density did not correlate with tissue eosinophilia.

The small peripheral bile ductules progressively proliferated so that by four weeks post-infection, adjacent portal tracts appeared linked together (Fig. 8). In both diet groups this continued until by eight weeks, parts of the liver were nodular, giving an impression of cirrhosis (Fig. 9). However, only one hamster (in the low protein diet group) developed a true cirrhosis, for in all others the nodularity was focal, related to the proximal worms (Fig. 5), the basic lobular architecture was not destroyed, and there was no accompanying fibrosis. A consistent difference between the two diet groups lay in the degree of ductular proliferation; in the high protein group this was mainly tubular, i.e. proliferation of ducts with lumens (Fig. 10) with some areas of cystic dilatation, but without significant encroachment on the liver parenchyma. Low protein fed animals, however, demonstrated progressive ductular proliferation until by twelve weeks parts of the peripheral liver substance had been obliterated by tubular (Fig. 10) and solid spindly celled ductules (Fig. 11) resulting in a macroscopic nodular appearance.

The expansion of central and peripheral portal tracts caused piecemeal necrosis of the limiting plates of hepatocytes and consequent accumulation of lipofuscin and haemosiderin pigment in Kupffer cells and macrophages of the portal tracts (Fig. 11); this process was more prominent in the low protein fed animals. Two hamsters from each diet group showed focal deposition of amyloid around portal tract vessels and proliferating bile ductules. As in the non-

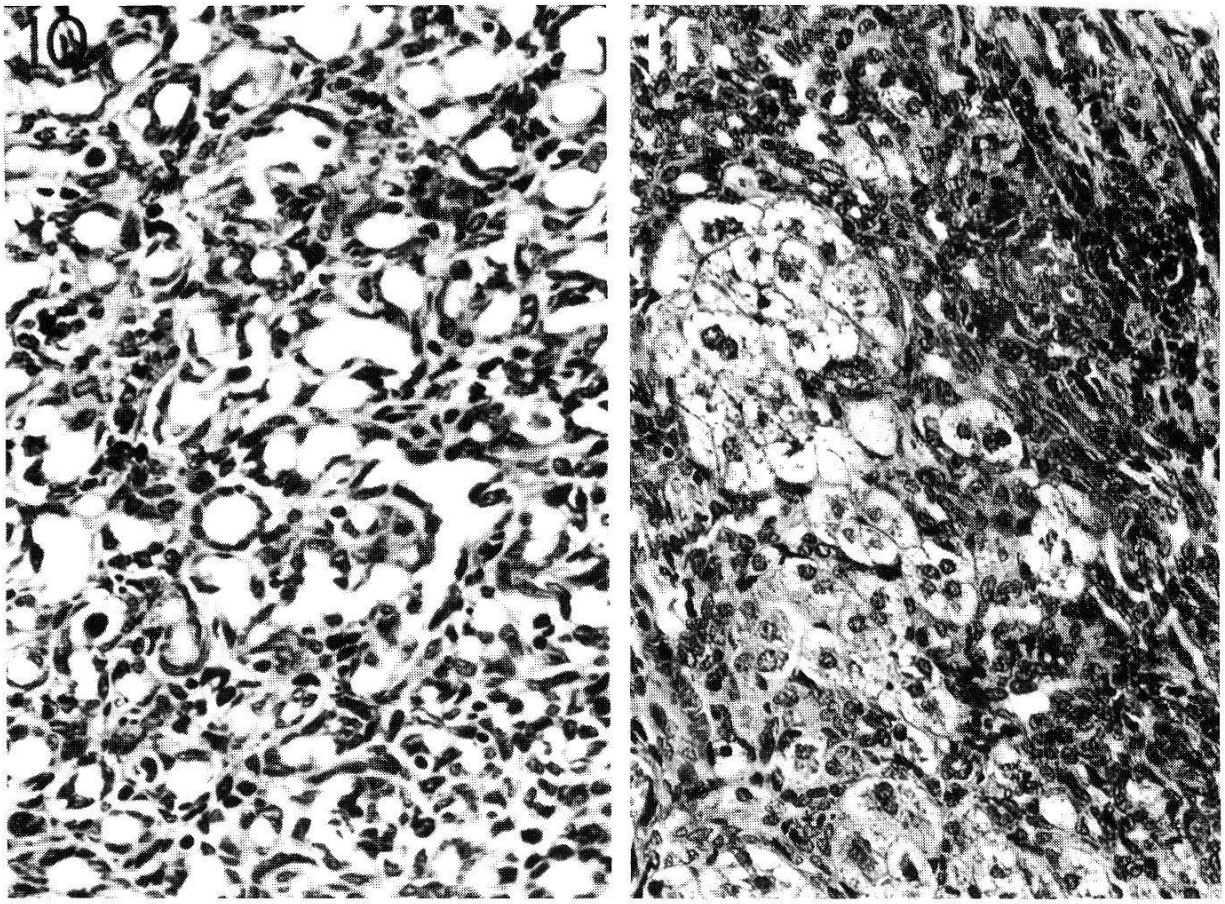

Fig. 10. L.P.D. hamster liver 12 weeks post-infection showing bile ductule proliferation with lumen formation (H & E, 350×).

Fig. 11. L.P.D. hamster liver 12 weeks post-infection showing piecemeal necrosis of vacuolated hepatocytes by proliferating spindle shaped bile duct epithelial cells (H & E, 250×).

infected control animals, low protein diet plus infection resulted in more focal hepatitis and cytoplasmic vacuolation than that observed in infected animals on the high protein diet. However, liver cell atrophy was less marked in the infected than the non-infected low protein fed animals.

Throughout the course of the experiment, no degenerate worms or host reaction to intraduct worms were seen. Although sequential parasite counts could not be performed on the livers, there was no histological impression of different worm loads between the two diet groups. One hamster in the low protein diet group had an acute cholangitis, with an intrahepatic stone of bile and ova but none of the animals showed other evidence of bile duct obstruction, i.e. cholestasis, periductal polymorph reaction and peripheral fibrosis.

## Discussion

Animal models of opisthorchiasis have been studied relatively little. Sun and Gibson (1969a, b) described infection with *C. sinensis* in mice, rats, guinea pigs and rabbits, however, only guinea pigs and rabbits have proved to be suitable laboratory hosts (Wykoff, 1958). Bhamarapravati et al. (1978) have

described the histopathological response of hamsters with *O. viverrini* infections. However, none of these studies have investigated the effects of dietary manipulation on the pathological response of the host to infection. This point may be of some importance to the outcome of *O. viverrini* infections in man, as the diet consumed by individuals in certain parts of north-east Thailand, where *O. viverrini* infection is endemic, is considered to be of a poor quality (Migasena, 1972).

The results of the present study show that the feeding of a low protein diet to hamsters does not impede the development of *O. viverrini* worms in the liver or delay significantly the period of maturation to egg release. On histopathological evidence, neither diet group of animals appeared to mount any form of rejection response against worms in the bile ducts, although the inevitable lack of a sequential parasite count precludes further conclusions.

Uninfected control hamsters maintained on the low protein diet showed evidence of liver cell atrophy, focal necrosis and accumulation of lipofuscin and iron pigment derived from cell breakdown. Infected animals from both dietary groups had piecemeal necrosis of limiting plates due to the expansion of portal tracts, but this was more severe in the low protein group due to the greater degree of bile ductule proliferation. This produced focal nodularity of the liver, but by modern criteria this is not classifiable as cirrhosis. Cholestasis or other evidence of bile duct obstruction was not seen in the infected animals, and it is not a common finding in other experimental models of opisthorchiasis.

Granuloma development around eggs that breached the ducts or ductular epithelium in animals from the present study, appeared unaffected in size and extent of necrosis by dietary protein restriction. The nature of the granuloma, i. e. foreign body or delayed hypersensitivity type (Type IV), is not ascertainable by histology alone. Protein deprivation is known to reduce the size of the delayed hypersensitivity type granuloma in schistosomiasis (Knauft and Warren, 1969), and the similarity in granuloma size in the high and low protein fed animals with opisthorchiasis in the present study suggests that delayed type hypersensitivity to the eggs is not operative. In this respect, studies by Sun (1969) and Sun and Gibson (1969a, b) have demonstrated that the eggs of *C. sinensis* are non-antigenic in the definitive host. Further studies regarding the immunogenicity of *O. viverrini* eggs are presently in progress.

Bhamarapravati et al. (1978) concluded that the resolution of periductal granulomas around eggs led to periductal scarring and fibrosis. The present study indicates that this is unlikely as concentric fibrosis had already commenced by two weeks of infection, before egg laying had commenced. Furthermore, the granulomas in the portal tracts were mostly situated outside the sleeve of periductal fibrosis, and, on hyalinising fused with the area of fibrosis. Moreover, it was observed that the extent of periductal fibrosis, but not granuloma development was affected by protein dietary restriction. It therefore seems more likely that the periductal fibrosis resulted as a direct consequence of the

flukes. Particularly during their growth and maturation period within the ducts (3–4 weeks), they dilated the ducts and caused focal necrosis of the epithelium with consequent access of bile and worm products to the portal tract tissues resulting in inflammation and fibrosis. The reduced periductal collagen in the livers of protein deprived infected animals may be ascribed to reduced fibroplasia seen in animals maintained on protein restricted diets (Pearce et al., 1960).

The inflammatory reaction in the early stages of infection was predominantly eosinophilic and lymphocytic, changing to plasma cell infiltration as the infection progressed. *O. viverrini* remains localised outside the tissues proper for the entire duration of its life history in the mammalian host in contrast to many other helminths that cause eosinophilia. Furthermore, the egg granulomas seen in the present study contained no eosinophils, unlike the egg granulomas of *Schistosoma mansoni* (Moore et al., 1977). Therefore the early maximal tissue eosinophilia seen in the present study may relate to the expansion of worms in the ducts causing damage to the mucosa and allowing an ingress of worm and bile products to the tissue spaces. Mast cell degranulation was not observed, and a correlation between mast cells and eosinophil density was not found, suggesting that immediate type hypersensitivity is an unlikely cause of the tissue eosinophilia. Sun and Gibson (1969a, b) have shown that serum from experimentally infected animals contains antibodies reacting principally with worm metabolic products rather than with parasitic somatic antigens, and the periductal plasma cell infiltrates observed in the present study probably represents production of these antibodies.

The cause of the biliary proliferation occurring peripherally (at a distance from the actual site of fluke lodgement) may possibly be due to worm-derived soluble factor(s) (Hou, 1956; Bhamarapravati et al., 1978). The marked increase in the severity and extent of bile ductule proliferation in animals from the low protein diet group may indicate an increased susceptibility of biliary epithelial cells in these animals to proliferate in response to a stimulus provided by the parasite. Hyperplasia of the epithelium adjacent to the worms has been found in all studies of opisthorchiasis. Bhamarapravati et al. (1978) have speculated that the excessive hyperplasia may be a result not just of epithelial repair of ulceration, but also of chemical products released by the worms. None of the hamsters in the present study, however, showed second order bile duct adenomatous hyperplasia.

The aetiological relationship between opisthorchiasis and cholangio-carcinoma is well supported by studies on the epidemiology (Gibson, 1971) and histopathology of *C. sinensis*. Similar epidemiological support has been presented for *O. viverrini* (Bhamarapravati and Vajrasthira, 1966; Sonakul et al., 1978). Hou (1964, 1965) reported cholangiocarcinomas in *C. sinensis* infected cats and a dog, whilst *O. viverrini* infected hamsters required in addition the carcinogen, dimethylnitrosamine before bile duct carcinomas developed (Thamavit et al., 1978).

In natural infections of *O. viverrini* and *C. sinensis* that result in cancer, the malignant change takes place in the second order bile ducts where the worms reside. In most cases the tumour develops from adenomatous hyperplasia in the bile duct wall (Hou, 1956). Hou also pointed out that cirrhosis, cholestasis and terminal bile ductule proliferation are not features of human opisthorchiasis. The present study has shown that *O. viverrini* in hamsters not only results in second order bile duct hyperplasia but also in proliferation of bile ductules but without any indication that either process is pre-neoplastic. The administration of dimethylnitrosamine to hamsters results in biliary proliferation leading eventually to cholangiocarcinoma (Tomatis et al., 1964; Herrold, 1967). Thamavit et al. (1978) have shown that the administration of dimethylnitrosamine to *O. viverrini* infected hamsters leads to the appearance of cholangiocarcinomas with a short latent period between carcinogen application and tumour appearance. Non-infected control animals receiving the same amount of carcinogen did not develop tumours over the same time period. However, in none of these studies is it clear where the cholangiocarcinoma originates, whether from the second order bile ducts as in infected cats, dogs and man or from the proliferating bile ductules which are peripheral to the site of worm lodgement. Reddy et al. (1977) administered 4-dimethylamino-3′-methylazobenzene (3′-MeDAB) to rats and observed cholangiocarcinomas and transitional stages between these tumours and islets of cholangiofibrosis. The pre-neoplastic cholangiofibrosis which they describe is similar to the bile ductule proliferation seen in animals in the present study. Furthermore, Flavell and Goepel (unpublished observations) have noted that rats fed 3′-MeDAB develop peripheral bile ductule proliferation preceeding the appearance of bile duct tumours.

Chesterman and Pomerance (1965) have described spontaneous liver nodularity, bile duct proliferation and some cholangiocarcinomas in hamsters fed on various non-carcinogenic diets and without evident helminthic infections, illustrating the lability of the hamster liver structures. None of the control animals from the present study developed these pathological features, but the similarity in the nodularity and bile duct proliferation between animals with opisthorchiasis and animals in the early stages of hepatocarcinogenesis points to the possibility of a similar pathogenetic process.

If infection with *O. viverrini* does lead to cholangiocarcinoma in the hamster, the tumour may then originate from the ductular proliferation. It is conceivable that opisthorchiasis renders the biliary epithelium more susceptible to the action of an environmental carcinogen present at "subthreshold" levels and therefore ineffective or less effective against uninfected individuals. In this respect Thamavit et al. (1978) have noted the possible sources of N-nitroso compounds in the diet of the north-eastern Thai. Thus, the failure to produce cholangiocarcinomas in infected animals from the present study may result from either a lack of a further carcinogenic stimulus or alternatively because the infection period was too short. Moreover, in the present study only one single

inoculum of metacercariae was administered to animals, whereas in natural infections of man continuous re-infection occurs each time a meal of raw fish is consumed. This continual barrage of reinfecting parasites may provide a further stimulus favouring tumourigenesis. Further studies are in progress to help establish the role played by these various factors in the pathogenesis of opisthorchiasis.

*Acknowledgments.* We gratefully acknowledge Professor Romsai Suwanik for allowing this work to be carried out in his department and also the staff of the Histology Department, Siriraj Hospital and Dr. Iti of the National Cancer Institute, Bangkok for help in processing tissue sections. We would also like to express our gratitude to Professor S. Vajrasthira for constructive advice and the United States Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok and the Animal House services of Chulalongkorn University for their gift of experimental animals. The assistance of Mr. V. Clark, St. Thomas' Hospital, London with photomicrographs is also gratefully acknowledged. – This work was financed in part by the Wellcome Trust and China Medical Board of New York Inc. DJF and SBL are Wellcome Research Fellows.

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