Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*)

A. A. CUNNINGHAM[1], T. E. S. LANGTON[2], P. M. BENNETT[1], J. F. LEWIN[3], S. E. N. DRURY[4], R. E. GOUGH[4] AND S. K. MACGREGOR[1]

[1] *Veterinary Science Group, Institute of Zoology, Regent's Park, London NW1 4RY, U.K.*
[2] *Herpetofauna Consultants International, Triton House, Bramfield, Halesworth, Suffolk IP19 9AE, U.K.*
[3] *The Royal Free Hospital School of Medicine, Pond Street, Hampstead, London NW3 2QG, U.K.*
[4] *Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, U.K.*

SUMMARY

In 1992 we began an investigation into incidents of unusual and mass mortalities of the common frog (*Rana temporaria*) in Britain which were being reported unsolicited to us in increasing numbers by members of the public. Investigations conducted at ten sites of unusual mortality resulted in two main disease syndromes being found: one characterized by skin ulceration and one characterized by systemic haemorrhages. However, frogs also were found with lesions common to both of these syndromes and microscopic skin lesions common to both syndromes were seen. The bacterium *Aeromonas hydrophila*, which has been described previously as causing similar lesions, was isolated significantly more frequently from haemorrhagic frogs than from those with skin ulceration only. However, as many of the latter were euthanased, this may have been due to differences in *post mortem* bacterial invasion. An iridovirus-like particle has been identified on electron microscopical examination of skin lesions from frogs with each syndrome and iridovirus-like inclusions have been detected in the livers of frogs with systemic haemorrhages. Also, an adenovirus-like particle has been cultured from one haemorrhagic frog. A poxvirus-like particle described previously from diseased frogs has now been found also in control animals and has been identified as a melanosome. Both the prevalence of the iridovirus-like particle and its association with lesions indicate that it may be implicated in the aetiology of the disease syndromes observed. Specifically, we hypothesize that primary iridovirus infection, with or without secondary infection with opportunistic pathogens such as *A. hydrophila*, may cause natural outbreaks of 'red-leg', a disease considered previously to be due to bacterial infection only.

1. INTRODUCTION

Incidents of unusual mortality of the common frog (*Rana temporaria*) were reported unsolicited to two of the authors (AAC & TESL) by members of the public at an increasing rate between 1985 and 1991. This period coincided with suggestions in the scientific literature of an apparent global decline in amphibian populations, including recent and unexplained extinctions of some amphibian species (see Cunningham *et al.* 1993). In 1992 a study (termed the Frog Mortality Project) was initiated in order to investigate the occurence of the mortality incidents in Britain. Initial epidemiological and pathological findings from this study, including those of negative contrast electron microscopy of fresh skin samples, were reported by Cunningham *et al.* (1993). Briefly, in 1992, 222 incidents of adult frog mortality were reported which were unusual either because of the number of frogs involved, or because of the lesions seen, or a combination of these. The main lesions reported were reddening of the skin and skin ulceration, with haemorrhagic gastro-enteritis and systemic haemorrhages also frequently being found on laboratory examination of carcasses.

Frogs found dead or dying with reddened skin and systemic haemorrhages have been described both in the wild and in captivity before (Reichenbach-Klinke & Elkan 1965; Nyman 1986; Bradford 1991) with reports dating back to at least the 1890s (Sanarelli 1891; Russell 1898). This condition has been termed 'red-leg' in recognition of the predominant clinical findings of erythema and haemorrhages of the hindleg skin (Emerson & Norris 1905). However, these findings are non-specific and other lesions, including skin ulceration, distal limb necrosis and gastro-intestinal haemorrhages, have also been described from frogs attributed as having 'red-leg' (Reichenbach-Klinke & Elkan 1965; Gibbs *et al.* 1966; Anver & Pond 1984) and this term is considered inappropriate by some authors (Gibbs *et al.* 1966).

In this paper we present detailed results of our pathological examinations, which include bacteriological and virological studies. Six frogs which had

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been killed by cats were collected in 1995 and used as controls for this study, as the nature of the funding for this work precluded the sacrifice and examination of healthy, control animals.

2. MATERIALS AND METHODS

(*a*) *Post mortem examinations*

Dead and diseased common frogs were collected for post mortem examination from ten disparate sites of unusual mortality in 1992 (tables 1 & 2), each site being a garden pond in the South East of England (Cunningham *et al.* 1993). Thirty-three carcasses were collected from eight sites and 20 moribund frogs were collected from six sites. The latter were euthanased on welfare grounds by stunning and pithing and 18 were examined within one hour of being killed. Collected carcasses were stored at +4 °C and were examined within 48 hours of the animals being found dead. Detailed systematic gross post mortem examinations were conducted and tissue samples were taken for further examinations. Although spawn, tadpoles and metamorphs also were found dead at some of the sites visited, all of the frogs necropsied were adult, but of varying sizes and weights (table 2).

In addition to the above, six common frogs from a garden pond in Surrey which were seen to be killed by cats were stored at +4 °C and examined post mortem within 72 hours of death. No unexplained deaths of frogs had been seen at this site prior to the cat predation, and the remaining frogs bred successfully during 1995 with no evidence of unusual mortality.

(*b*) *Histological examinations*

A wide range of tissues, including samples of all major organs and femoral skin and muscle, and of skin from areas of ulceration and from areas of distal limb necrosis, was collected from each frog examined and fixed in neutral buffered 10% formalin. Fixed material was processed, embedded in paraffin wax, sectioned for histological examination and stained with haematoxylin and eosin using standard methods. Bone was decalcified using Ethylene Diamine Tetra-Acetic acid prior to processing. Sections of grossly normal femoral skin were scrutinized from all frogs necropsied. Other tissues were examined histologically from a minimum of three frogs from each site of mortality, except for Site 3 from which the tissues of two frogs only were examined.

(*c*) *Microbiological examinations*

A range of tissues, including liver, heart blood, tongue, stomach, intestine, femoral skin and femoral muscle, were taken using sterile techniques for routine bacteriology from most of the frogs. Also, bacteriological examinations were carried out on lung (18 frogs); kidney (16); areas of skin ulceration (9); areas of distal limb necrosis (4) and on swabs from erythematous, non-ulcerated skin (42). A more limited range of tissues was taken for bacteriology from three frogs which appeared not to be freshly dead: stomach and intestine only was sampled from one frog (ref. 435/92), liver only from one frog (336/92) and oviduct only from one frog (520/92). Liver, kidney, intestine, and femoral muscle were examined bacteriologically from three control frogs necropsied within 24 hours of death and liver, kidney and femoral muscle were examined from one control frog examined within 48 hours of death. Tissue samples were plated onto 5% horse blood agar and onto xylose lysine desoxycholate agar and were incubated aerobically at 25 °C for 24 and 48 hours. Necrotic tissue from an area of hindlimb necrosis from one frog (339/92) was cultured anaerobically at 25 °C for 24 and 48 hours on 5% horse blood agar. Bacterial isolates were identified using API biochemical test strips (API-bio Merieux (UK) Limited, Basingstoke, Hampshire, United Kingdom). Further biochemical tests were carried out on two isolates cultured from frogs collected from Site 1 by the Central Public Health Laboratory, Colindale. Erythematous skin (from 5 frogs), lung (2), tongue (2), an area of skin ulceration (1) and necrotic tissue from an area of hindlimb necrosis (1) were plated onto Sabouraud's agar and incubated at 25 °C for 7 days. In addition, smears were made from skin ulcers from three frogs and stained with Ziehl-Neelsen's stain.

Table 1 *Incidents of unusual mortality of common frogs* (Rana temporaria) *investigated in* 1992

site	location	month incident		duration of mortality (weeks)	no. frogs found dead*
		started	finished		
1†	Sussex	April	August	20	8
2	Surrey	February	November	40	156
3	Essex	July	September	12	27
4	Hampshire	August	September	8	90
5	Surrey	July	October	16	139
6	Essex	August	September	8	22
7	Middlesex	August	September	8	40
8	Hampshire	July	October	16	110
9	Essex	August	October	12	15
10	Dorset	August	September	8	10

* Does not include euthanased frogs. † Approximately 40 frogs had been found dead at this site over a five month period during the previous summer (1991).

(*d*) *Virological examinations*

Samples of skin from the femoral region, taken from 50 diseased frogs and stored frozen at -70 °C, were thawed, processed using standard techniques and examined using negative contrast electron microscopy (EM) (Cunningham *et al.* 1993). Samples of skin from the femoral region of six control frogs were also examined. Additionally, poxvirus-like particles were pelleted (by microcentrifugation at 15200 g for 2 minutes of fluid from processed skin positive for poxvirus-like particles on EM), fixed in 2.5% glutaraldehyde and processed using standard techniques for examination using transmission electron microscopy (TEM).

Skin samples from the edges of ulcers from six frogs examined within one hour of euthanasia and from one fresh carcass, were cut into 1 mm squares, fixed in 2% glutaraldehyde solution, processed using normal methods and examined using TEM. Additionally, areas of skin with apparent epidermal hyperplasia from three frogs (449/92, 460/92 & 487/92), areas of liver containing inclusion bodies from three frogs (458/92, 465/92 & 527/92), and an area of spleen containing focal necrosis from one frog (445/92) processed for histology were cut out of paraffin blocks and processed for TEM using the methods of Lewin *et al.* (1995).

Supernatants of homogenated fresh (frozen/thawed) femoral skin (suspended 20% w/v in sterile phosphate buffered saline containing 1% penicillin/streptomycin, 0.05% gentamicin and 0.5% nystatin) from 32 frogs were inoculated onto cultures of a commercially available frog (*Rana pipiens*) embryo fibroblast cell line (ICR-2A) obtained from the European Collection of Animal Cell Cultures. Following inoculation, the cultures were incubated at 27 °C. Fifteen of these cultures were passaged twice, each passage lasting seven days, while 17 were passaged once only. Cultures in which a cytopathic effect (CPE) was seen were processed and examined using direct negative contrast EM as described by Drury *et al.* (1995).

3. RESULTS

(*a*) *Post mortem examinations*

(*i*) *Haemorrhagic syndrome*

In 20 of the frogs examined, the predominant finding was of systemic petechial and ecchymotic haemorrhages, primarily within the skeletal musculature and the alimentary and reproductive tracts (see below) (figure 1). Although intramuscular haemorrhages were seen in the hindlegs only in seven of these frogs, in the majority of cases intramuscular haemorrhages also were present elsewhere in the body. Intramuscular haemorrhages were not seen in three frogs which were classified as having this haemorrhagic syndrome (HS).

(*ii*) *Ulcerative skin syndrome*

In 19 of the frogs examined, the predominant lesion was dermal ulceration with or without necrosis of one or more of the distal limbs (figure 2). In 15 of these animals there was extensive ulceration of the skin, primarily over the femora (figure 2), although often the forelimbs and other areas of the body were also similarly affected. Ten frogs with this ulcerative syndrome (US) had necrosis of one or more of the distal digits, often with distal phalanges missing or with these bones exposed, while in a further five frogs, the necrosis was more extensive: to the level of the tarsus in four frogs and to the level of the distal left femur in one frog. Four of these animals were found alive with bones protruding through the remaining necrotic soft tissues (figure 2*b*). Apart from one frog which had haemorrhages in the femoral musculature, two frogs with erythematous femoral musculature, one frog with erythema of the testes and one frog with erythema of the oviducts, none of the frogs with US had systemic haemorrhages or erythema (table 2), although in all cases there was reddening of exposed muscle following dermal ulceration.

(*iii*) *Ulcerative and haemorrhagic syndrome*

At three sites where mortality was investigated, frogs were found with lesions common both to US and to HS and therefore these animals will be referred to as the U+HS group (table 2). Of the seven frogs with U+HS, five had only small areas of dermal ulceration: typically one or two ulcers of 1–4 mm diameter over the hindlimbs or ventrum. In these cases, there often was a 2–3 mm zone of grey discoloration around the dermal ulcer (figure 3), this apparently being an area of epidermal ulceration only. Such a zone was seen in only one frog with US.

(*iv*) *Reddened-skin syndrome*

Five of six frogs necropsied from one incident (Site 1) had skin erythema only without skin ulceration or systemic haemorrhages, although one of these frogs (177/92) had erythema and petechiation of the caudoventral femoral musculature. These animals were categorized as the RS group (for reddened-skin syndrome). All frogs euthanased at this site were thin and lethargic with marked, diffuse reddening of the limbs.

(*v*) *Other lesions*

Erythema of the skin was noted in 26 of the frogs examined from across the four categories of primary lesion. The pattern of reddening was either of the hindlimbs only (7 frogs); the forelimbs only (3); all four limbs only (8); the ventrum only (1); a combination of the ventrum and limbs (4) or of the whole body including the limbs (3). Twenty-five frogs had no evidence of diffuse reddening of the skin, of these 14 were frogs with US, but nine frogs with HS and two frogs with U+HS also had no skin erythema. Subcutaneous oedema (possibly due to distention of the subcutaneous lymph sacs) was recorded in five frogs with HS and in two frogs with U+HS.

Fat stores were absent from 11 of the 19 frogs with US, from one frog with HS and from all five frogs with RS. However, the fat bodies found often were very small. Six frogs with US had marked wasting of the skeletal musculature and were judged as being em-

Table 2. *Main pathological findings in common frogs* (Rana temporaria) *from sites of unusual mortality*

mortality incident	frog ref. No.	sex	weight (g)	main lesion	skin erythema	gross haemorrhages			necrosis seen on histological examination				*A. hydrophila* from		liver virus inclusions	iridovirus-like particles seen in skin on TEM
						muscle	GIT	tongue	epidermis	liver	RMT	spleen	frog	viscera†		
Site 1 (Sussex)	172/92*	M	18	R	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NE
	173/92*	M	28	U	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG
	174/92*	M	22	R	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NE
	175/92*	M	20	R	POS	NEG	NEG	NEG	NEG	NEG	NEG	NE	NEG	NEG	NEG	NE
	176/92*	M	20	R	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NE
	177/92*	M	33	R	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NE
Site 2 (Surrey)	291/92	F	6	U	POS	NEG	NEG	NEG	NEG	NE	NE	NE	POS	NEG	NE	NEG
	292/92	F	10	U	NEG	NEG	NEG	NEG	NEG	NE	NE	NE	POS	NEG	NEG	NE
	293/92	M	9	U	NEG	NEG	NEG	NEG	NE[1]	NE	NE	NE	POS	NEG	NE	NE
	336/92	F	22	U	POS	NEG	NEG	NEG	NE	NEG	NE	NE	NEG	NEG	NEG	NE
	338/92*	F	18	U	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	POS
	339/92*	F	7	U	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NE
	340/92*	M	6	U	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG
	341/92*	M	13	U	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	POS	NEG	NEG	POS
	439/92*	M	22	U	NEG	NEG	NEG	NEG	NEG	NE	NE	NE	POS	NEG	NE	POS
Site 3 (Essex)	435/92	M	23	U	NEG	NEG	NEG	NEG	NE	NE	NE	NE	NEG	NE	NE	NE
	436/92*	M	39	U	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	NEG	POS
	440/92	F	40	H	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	NEG	NE
Site 4 (Hampshsire)	445/92*	M	21	U	POS	POS	NEG	NEG	NEG	POS	POS	POS	POS	POS	NEG	NE
	446/92	M	28	H	NEG	POS	POS	POS	POS	NE	NE	NE	POS	POS	NE	NE
	447/92	F	44	H	POS	POS	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	NE
	448/92	F	31	H	POS	POS	POS	POS	POS	NE	NE	NE	POS	NEG	NE	NE
	449/92	M	28	H	POS	POS	POS	POS	NE[1]	NEG	POS	NEG	POS	POS	NEG	POS
Site 5 (Surrey)	457/92	F	83	H	POS	NEG	POS	NEG	NE[1]	NE	NE	NE	POS	POS	NE	NE
	458/92	M	21	H	NEG	POS	POS	POS	POS	NEG	POS	POS	POS	POS	NEG	NE
	459/92	F	22	H	NEG	POS	POS	NEG	POS	NEG	POS	POS	POS	POS	NEG	NE
	460/92	F	41	H	NEG	POS	POS	POS	POS	NEG	POS	POS	POS	POS	POS	POS
Site 6 (Essex)	464/92	M	30	U+H	NEG	POS	POS	NEG	NE[1]	POS	POS	POS	POS	NEG	POS	NE
	465/92	F	42	U+H	NEG	POS	POS	NEG	POS	NEG	NE[2]	POS	NEG	NEG	POS	NE
	466/92	M	29	U+H	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	NE
	467/92	F	37	U+H	POS	POS	NEG	POS	POS	NE	NE	NE	POS	POS	NE	NE
	468/92	M	34	H	POS	POS	NEG	NEG	POS	NE	NE	NE	POS	NEG	NE	NE
Site 7 (Middlesex)	482/92	M	30	H	POS	POS	NEG	POS	POS	NEG	POS	POS	POS	POS	POS	NE
	483/92	F	55	U+H	POS	POS	NEG	NEG	NE[1]	NEG	NE[2]	NEG	NEG	NE	NEG	NE
	484/92	M	26	H	POS	POS	NEG	NEG	NE[1]	POS	POS	POS	POS	POS	POS	NE
	485/92	M	36	H	POS	NEG	NEG	NEG	NE[1]	NE	NE	NE	POS	POS	NE	NE
	486/92	M	42	H	NEG	POS	NEG	NEG	POS	NE	NE	NE	NEG	NEG	NE	NE

Site 8	487/92*	M	15	U	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
(Hampshire)	488/92*	M	15	U	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NE
	489/92*	F	6	U	NEG	NEG	NEG	NE	NE	NE	NEG	NEG	NE	NE
	490/92*	F	23	U	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NE
	491/92*	F	29	U	NEG	NEG	NEG	NE	NE	NE	POS	NEG	NE	NE
Site 9	518/92	F	23	U+H	POS	NEG	POS	NEG	POS	NEG	POS	NEG	NEG	NE
(Essex)	519/92	M	40	U+H	POS	POS	POS	POS	POS	NEG	NEG	NEG	POS	NE
	520/92	F	44	H	NEG	NEG	NEG	NE	NE	NE	NEG	NE	NE	NE
	521/92*	F	64	U	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NE
Site 10	523/92	M	28	H	POS	POS	NEG	NE[1]	NEG	POS	POS	POS	NEG	NE
(Dorset)	524/92	M	25	H	POS	POS	NEG	POS	POS	POS	NEG	POS	NEG	NE
	525/92	M	31	H	NEG	POS	NEG	NE[1]	NE	NE	NE	POS	NE	NE
	526/92	M	37	H	NEG	POS	NEG	POS	NE	NE	NE	POS	NE	NE
	527/92	F	63	H	NEG	POS	NEG	POS	NEG	POS	POS	POS	POS	NE

Key: * = euthanased, R = skin erythema, U = skin ulceration, H = systemic haemorrhages, NEG = negative, POS = positive, NE = not examined, 1 = epidermis sloughed from dermis and not present on section, 2 = autolysed, RMT = renal myeloid tissue, † = viscera not including the GI tract.

Figure 1. Ventral view of a frog (ref. 485/92) with haemorrhagic syndrome. The ventral body wall and limbs have been removed to reveal the viscera. Note diffuse erythema throughout the viscera, and particularly the haemorrhagic gut (g) and oviduct (o).

aciated (figure 4). Four of these frogs were from Site 2 and two were from Site 8. There were petechial and ecchymotic haemorrhages throughout the fat bodies of two frogs with U+HS and of three frogs with HS.

Gross signs of enteritis or gastro-enteritis, as assessed by erythema, congestion or a combination of these, were seen in three of 19 frogs with US, all 20 frogs with HS, all seven frogs with U+HS, and one of five frogs with RS. The lesions in the frogs with US and with RS were mild, consisting of slight inflammation of a region of the intestine only, whereas the lesions in the frogs with HS and U+HS were more extensive and severe with petechiation of the gut wall. Of the frogs with gastro-enteritis, 11 with HS and three with U+HS had frank haemorrhaging into the lumen of the gastro-intestinal (GI) tract. Erythema of the tongue, with or without petechial and ecchymotic haemorrhages, was seen in 18 frogs (10 with HS, 5 with U+HS & 3 with US). In these animals the tongue was often ulcerated and haemorrhagic (table 2) and in some cases there were petechial haemorrhages and erythema of other areas of the oral mucosa. The muco-cutaneous junction of the anus was markedly haemorrhagic in eight frogs (5 with HS & 3 with U+HS).

The pancreas of one frog with HS and of two frogs with U+HS contained ecchymotic haemorrhages and the caudal half of the pancreas of one frog with U+HS was blackened and necrotic.

The lungs were congested in 17 frogs (5 with US, 7 with HS, 2 with U+HS & 3 with RS). Five frogs with HS and three with U+HS had congestion of the liver. Two frogs with US, five with HS and two with U+HS had congested kidneys. The urinary bladder was noted to be congested in two frogs with U+HS from Site 6 and to be congested and haemorrhagic in one frog with U+HS from Site 9.

The spleens of frogs with systemic haemorrhages (frogs with HS and U+HS) were congested and enlarged (mean size of 5.0×4.8 mm, $n = 26$) when compared with those of frogs without haemorrhages (frogs with US and RS) (mean size of 4.0×3.8 mm, $n = 14$). However, the significance of these figures should be interpreted with caution because of possible confounding factors such as the age and size of the frogs

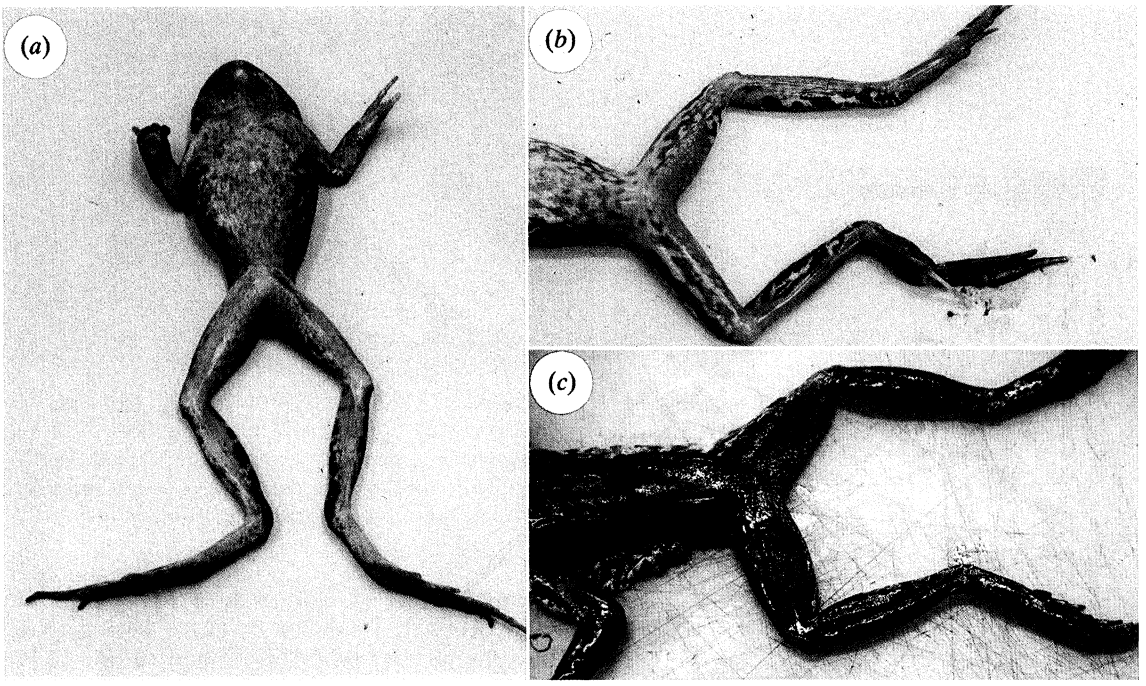

Figure 2. Main lesions of frogs with ulcerative syndrome. (a) Ventral view of frog ref. 338/92 with a linear femoral ulcer and severe ulceration of the right forefoot with loss of digits. (b) Frog ref. 339/92 with necrosis of the right hindfoot. (c) Frog ref. 439/92 with extensive skin ulceration of the dorsal aspect of the left thigh.

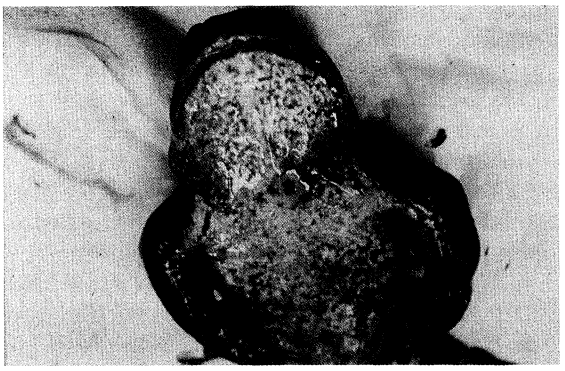

Figure 3. Ventral view of a frog (ref. 464/92) with ulcerative and haemorrhagic syndrome. Note the small central area of dermal ulceration surrounded by a grey zone of epidermal ulceration.

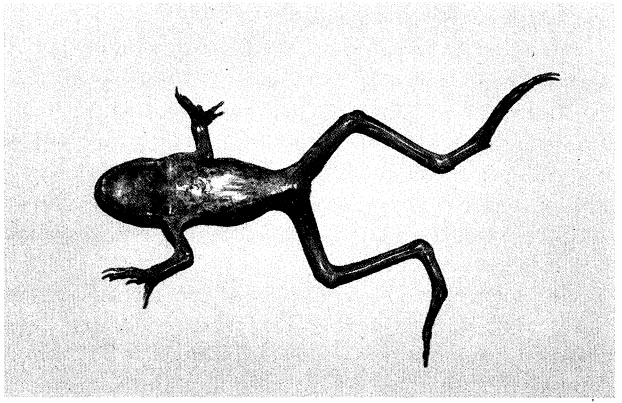

Figure 4. Ventral view of an emaciated frog (ref. 291/92) with ulcerative syndrome. Note the marked wasting of skeletal muscle and skin ulcers over the right thigh, left tarsus and throat.

examined. Multiple small (< 0.5 mm diameter), white foci were seen throughout the spleens of three frogs with HS and of two with U + HS.

Thirty of the frogs examined were male, of which one of ten with US, eight of 12 with HS, all three with U + HS, and none of five with RS had erythema, petechiation, or a combination of these, over the surfaces of the testes. Of the 21 females examined, one of nine with US, six of eight with HS and three of four with U + HS had diffuse erythema, petechiation, or a combination of these, of the oviducts (figure 1).

Many of the frogs examined contained variable, usually low, burdens of helminth parasites. In no case was parasitism regarded as contributory to the cause of death. These findings will be presented in detail elsewhere.

(vi) Control frogs

All of the control frogs examined had been killed by being bitten through the head and anterior body. Bitewounds and associated damage were often evident elsewhere over the body and limbs. None of these animals had macroscopic lesions other than those directly attributable to predation by cats, and none of these lesions resembled those seen in frogs with any of the above four disease syndromes.

(b) *Histological examinations*

(i) Diseased frogs

Femoral skin and muscle was examined histologically from 47 of the 51 frogs necropsied. The epidermis of nine of these frogs (1 with US, 2 with U + HS & 6 with HS) could not be examined as it had sloughed from the dermis and was not present on the sections (table 2). In

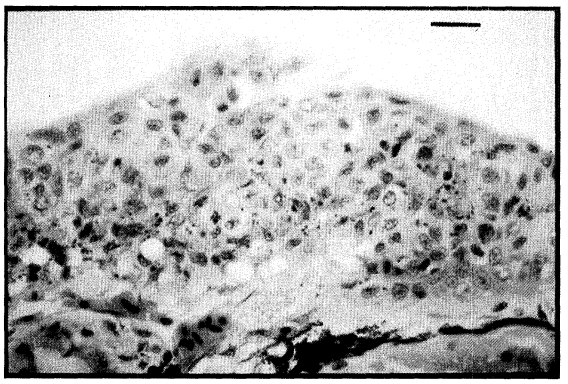

Figure 5. Photomicrograph of femoral skin from a frog (ref. 460/92) with haemorrhagic syndrome showing epidermal thickening with necrosis of the deeper cell layers. H&E. Scale Bar = 25 μm.

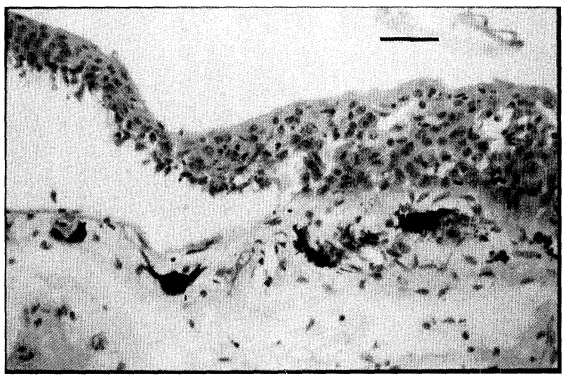

Figure 6. Photomicrograph of femoral skin from the same frog as figure 5 showing separation of the epidermis from the dermis. H&E. Scale Bar = 50 μm.

these cases the epidermal loss was recent as the exposed dermis appeared to be healthy. Skin from other areas of the body was examined from the remaining four frogs.

Generally, the epidermis was 3–6 layers of cells thick in all frogs examined, but areas where the epidermis was 6 to 10 cells thick were seen in seven frogs (2 with RS, 2 with US, 1 with U+HS & 2 with HS) and plaque-like regions or papillary structures where the epidermis comprised between 10 and 30 layers were seen in an additional 26 frogs (3 with RS, 11 with US, 4 with U+HS & 8 with HS). In many of these areas there was necrosis of the epidermis, most notably of the deeper cells (figure 5). Additionally, extensive areas of necrosis were seen in the epidermis examined in four of five frogs with U+HS and in 13 of 14 frogs with HS. The predominant lesion was necrosis of the stratum basale and the stratum spinosum, and often there was separation of the epidermis from the dermis (figure 6).

Areas of skin ulceration were examined histologically from five frogs with US and from three frogs with U+HS. Also, areas of distal limb necrosis were similarly examined from three frogs with US. In all cases the findings were of dermal ulceration with necrosis and bacterial invasion of the exposed muscle and soft tissues. Bone necrosis also was seen in the cases examined with distal limb necrosis. Infiltration by

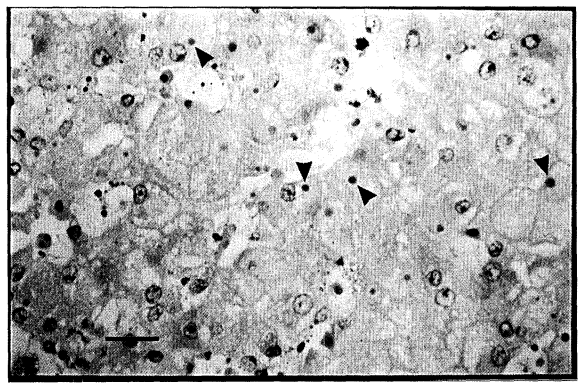

Figure 7. Photomicrograph of liver from a frog (ref. 465/92) with ulcerative and haemorrhagic syndrome showing intracytoplasmic basophilic inclusions (arrowheads) within hepatocytes. H&E. Scale Bar = 25 μm.

granulocytes and lymphocytes within affected tissues and surrounding muscle and dermis, often with congestion, oedema and focal haemorrhaging within these tissues, was also a consistent finding.

On histological examination, the lungs were either normal (10 frogs), congested only (11), or were congested with haemorrhage, focal acute necrosis, infiltration and exudation of granulocytic inflammatory cells, or a combination of these (11). The lungs from one frog contained haemorrhaged blood. There was no apparent association between the type of pulmonary lesions and the disease syndrome of the frogs. Lungworms were present in nine of 11 lungs with pneumonia, two of 11 lungs with congestion only and in five of 10 lungs with no lesions.

The livers of 31 frogs were examined histologically. A marked increase in the numbers of melanomacrophages was observed in eight livers (1 frog with RS, 3 with US, 2 with U+HS and 2 with HS). Focal acute necrosis of hepatocytes was seen in seven frogs (2 with US, 2 with U+HS and 3 with HS). In one of these frogs (445/92) the necrosis was locally diffuse. In one frog (436/92) there were multinucleated hepatocytes and mitotic figures, possibly indicating hepatocellular regeneration. Basophilic intracytoplasmic inclusions (figure 7) were seen in the hepatocytes of nine frogs (5 with HS & 4 with U+HS) (table 2). Also, intracytoplasmic acidophilic inclusions (figure 8) were seen in two of these frogs (484/92 & 527/92) and in one frog (458/92) without basophilic inclusions. Acidophilic bodies were surrounded by a clear halo, whereas basophilic inclusions were not.

Of 32 hearts examined histologically, no abnormalities were detected in 25. The remainder had pericardial inflammation (2 frogs with RS); focal myocardial necrosis (1 with US); focal myocardial inflammation (1 with HS); congestion with intramyocardial haemorrhage (1 with HS & 1 with U+HS), and myocardial congestion only (1 with HS).

The spleens of 29 frogs (12 with US, 9 with HS, 4 with U+HS & 4 with RS) were examined histologically. On histological examination, the white foci seen grossly were shown to be areas of acute necrosis. Such foci, predominantly karyorrhexis of the lymphocytes, were observed in the spleens of 12 frogs (1

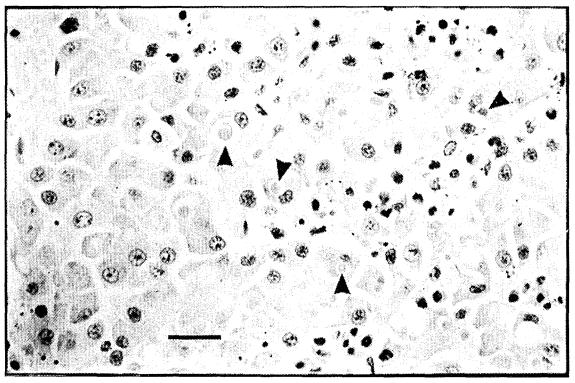

Figure 8. Photomicrograph of liver from a frog (ref. 458/92) with haemorrhagic syndrome showing intracytoplasmic acidophilic inclusions (arrowheads) within hepatocytes. H&E. Scale Bar = 25 μm.

with US, 9 with HS & 2 with U + HS). A granulocytic inflammatory response was associated with the necrotic foci in four frogs with HS, one frog with US and four with U + HS. Also, congestion and apparent lymphoid depletion were common findings: The spleens of 14 frogs (5 with HS, 3 with US, 4 with U + S & 2 with RS) were congested, while the spleens of 14 frogs (5 with HS, 2 with US, 2 with U + HS & 1 with RS) showed evidence of lymphoid depletion when compared with the histological appearance of the remaining spleens.

Subcapsular and interstitial myelopoiesis (predominantly granulopoiesis) was present in all kidneys examined histologically. There was necrosis of this tissue in two of 11 frogs with US, all 11 frogs with HS, all four frogs with U + HS and in none of five frogs with RS. Focal glomerular or tubular necrosis was seen in kidneys from four frogs with HS, one frog with U + HS and from two frogs with RS. The kidneys of two frogs with HS and one frog with RS contained one or more granulomata, consisting of focal infiltrates of macrophages and lymphocytes (with a smaller number of granulocytes) encapsulated by fibrous tissue. Two frogs with RS which did not have necrotic foci had areas of apparent mild nephritis with exudation of inflammatory cells into the renal tubules. One frog with US, four frogs with HS and four frogs with U + HS had congestion of the kidneys. Basophilic intracytoplasmic inclusion-like bodies were seen in a small number of tubular epithelial cells in the kidney of one frog with HS (527/92).

The three urinary bladders noted to be congested grossly were examined microscopically, as were two bladders which were grossly normal (449/92 & 464/92). All five bladders were congested and, in bladders (two grossly congested, one grossly normal) from three frogs, foci of epithelial necrosis with associated infiltrates of granulocytes were seen.

Grossly abnormal tongues were examined histologically from two frogs with U + HS and from four frogs with HS. In all cases there was a combination of acute epithelial necrosis with or without ulceration; granulocytic inflammation; congestion; intramuscular and submucosal haemorrhaging, and, in two cases, intramuscular oedema. There was bacterial overgrowth of the ulcerated areas.

The GI tract was preserved well enough to be examined histologically from 26 frogs (5 with RS, 10 with US, 4 with U + HS & 7 with HS). Of these, 13 frogs (5 with RS, 7 with US & 1 with HS) had evidence of mild focal infiltrates of granulocytes and lymphocytes within the mucosa and submucosa of the GI tract associated with the presence of nematodes in the lumen, crypts or submucosa. Occasionally, parasitic granulomata were seen in the submucosa. In four frogs with U + HS and five frogs with HS there was a much more severe gastro-enteritis, characterized by transmural congestion; submucosal oedema; focal necrosis of the mucosa; haemorrhages within the mucosa, submucosa and muscularis, and marked infiltrates of granulocytes and lymphocytes within the submucosa and mucosa. In two of these frogs (449/92 & 464/92) there were areas of intestinal ulceration with bacterial invasion and an exudate of granulocytes within the lumen. The two frogs with HS but without severe gastro-enteritis had very congested GI tracts.

The pancreases noted grossly to have lesions (including the cranial half of the blackened, necrotic pancreas) were found to be congested with multiple foci of necrosis, granulocytic inflammation and haemorrhage on histological examination. Of the pancreases examined from a further 16 frogs, no lesions were detected in 10 (1 with RS, 7 with US & 2 with HS), four (2 with HS & 2 with U + HS) were congested only and two (from frogs with HS) were congested with interstitial oedema and foci of necrosis and inflammation.

Testes were examined histologically from 21 frogs (5 with RS, 7 with US, 3 with U + HS & 6 with HS). Of these, the testes from three frogs (1 with U + HS & 2 with HS) were congested. No other lesions were detected. The oviducts were examined from 12 frogs (4 with US, 3 with U + HS & 5 with HS). The oviducts of one frog with US and of one frog with U + HS had granulocytic infiltrates within the serosa. There was marked congestion of the oviducts from two frogs with U + HS and from two frogs with HS. Multiple haemorrhages were seen within the mucosa of the oviducts from one frog with U + HS and from one frog with HS.

Fat bodies with gross haemorrhages were found to be congested with multiple foci of necrosis, granulocytic inflammation and haemorrhage when examined histologically.

(ii) Control frogs

Generally, the epidermis was 3 to 7 cells thick, but two frogs had areas 8 to 11 cells thick. Histological sections of lung examined from three of four control frogs contained focal, mild, interstitial infiltrates of granulocytes and lymphocytes. No other microscopic abnormalities were detected, but a slight to mild degree of subcapsular, with or without interstitial, myelopoiesis (predominantly granulopoiesis) was seen in the kidneys of all four frogs for which this organ was examined histologically.

Table 3. (*a*) *Bacteria isolated from frogs examined from incidents of unusual mortality.*

bacterium	no. of tissues	no. of frogs	no. of incidents
Aeromonas hydrophila	123	38	10
Flavobacterium breve	47	19	8
Acinetobacter sp.	41	19	7
Aeromonas sobria	38	12	5
Citrobacter freundii	28	17	9
Flavobacterium sp.	23	8	4
Pseudomonas putrefasciens	23	12	6
Pseudomonas sp.	21	11	4
Escherichia coli	16	12	8
Pseudomonas fluorescens	14	6	3
Enterobacter agglomerans	12	4	3
Bacillus sp.	12	7	4
Flavobacterium indologenes	10	4	2
Vibrio vulnificus	10	7	4
Hafnia alvei	9	7	5
Proteus sp.	9	7	4
Corynebacterium aquaticum	7	3	1
Buttiauxella aggrestis	4	4	3
Enterobacter sp.	4	4	3
Streptococcus group D	4	2	1
Streptococcus sp.	4	4	4
Vibrio sp.	4	3	3
Pseudomonas chloraphis	3	1	1
Pseudomonas putida	3	1	1
Escherichia vulneris	3	3	3
Aeromonas sp.	2	2	2
Enterobacter aerogenes	2	2	2
Enterobacter amnigenus	2	1	1
Vibrio metshnikovii	2	2	2
CDC gp 4 c 2	1	1	1
Cellulomonas turbata	1	1	1
Citrobacter diversus	1	1	1
Corynebacterium sp.	1	1	1
Morganella morgannii	1	1	1
Providencia stuartii	1	1	1
Pseudomonas cepacia	1	1	1
Pseudomonas vesicularis	1	1	1
Serratia marsescens	1	1	1
Staphylococcus epidermidis	1	1	1

(*b*) *Bacteria isolated from control frogs killed by cats*

bacterium	no. of tissues	no. of frogs	no. of incidents
Aeromonas hydrophila	11	4	1
Bacillus sp.	1	1	1
Corynebacterium aquaticum	1	1	1
Hafnia alvei	3	2	1
Pseudomonas sp.	5	3	1
Pseudomonas cepacia	1	1	1
Vibrio parahaemolyticus	1	1	1

(*c*) *Microbiological examinations*

For any given animal, the bacterial flora at different levels of the GI tract was very similar. Therefore, to aid analysis, results from these sites were amalgamated. Also, where more than one skin ulcer (including areas of distal limb necrosis) was examined from the same animal, the results were pooled. The same was done if more than one area of skin from one animal was sampled for bacterial culture.

The bacteria isolated and the number of times each organism was cultured from incidents of mortality, from the frogs necropsied and from the tissues sampled are listed in table 3. Forty bacterial isolates were identified. In some cases the species of bacterium could not be determined and the genus only is given. The frequency of isolation of the six species and four genera of bacteria which were found in the majority of incidents investigated are presented in tables 5*a* & *b* as a percentage both of the frogs and of the tissues sampled. *Aeromonas hydrophila* was isolated much more frequently than any other species and was the only bacterium to be found in frogs from all ten incidents of unusual mortality investigated (table 3). This bacterium also was cultured from control frogs. The percentages of tissues sampled, broken down by disease syndrome and by whether the frog was euthanased or found dead, from which *A. hydrophila* was isolated are listed in table 4*a*. Similarly, the percentages of tissues sampled from which no bacteria were grown are presented in table 4*b*. Over two thirds of the isolates of *A. hydrophila* from diseased frogs were made from the skin (20% of isolates), muscle (15%), tongue (18%) and GI tract (15%). This pattern of distribution was broadly similar for frogs with HS, US and U + HS, and both for frogs which had been found dead and for frogs which had been euthanased. *Aeromonas hydrophila* was isolated three times only from frogs with RS: once each from the skin, the tongue and the GI tract, the last two isolates being from the same frog. *Aeromonas hydrophila* was cultured from 90% of the frogs with HS, 74% of frogs with US, 57% of frogs with U + HS, 40% of frogs with RS and from all four control frogs sampled (table 5*a*).

As with *A. hydrophila*, the other commonly-isolated bacteria were most frequently cultured from the GI tract, tongue, skin and muscle. The pattern of isolation of *Acinetobacter sp.* was different, however, with this organism being cultured mostly from samples of skin (14% of *Acinetobacter sp.* isolates), muscle (20%) or tongue (20%) and less commonly (5%) from the GI tract. Also, this organism was isolated from five (50%) of ten skin ulcers sampled from frogs with US, but from only one (8%) of 13 non-ulcerated skin samples examined from these frogs.

Pure bacterial cultures were isolated from nine euthanased frogs (17 tissues) and from six diseased frogs found dead (9 tissues), but from none of the tissues sampled from control frogs. No organism was identified significantly more frequently as a pure culture than any other, but *Aeromonas spp.* were found in pure growth from the viscera only from animals with systemic haemorrhages (3 frogs, 5 tissues) and *Acinetobacter sp.* was isolated in pure growth only from animals with skin ulceration (6 frogs, 10 tissues).

Bacteriology was performed on the non-GI viscera (ie. lung, liver, kidney, heart, spleen, oviduct, fat body, urinary bladder, or a combination of these) from 51 diseased frogs (table 2) and from four control frogs.

Table 4. (*a*) *Percentage of frogs sampled, by organ and disease syndrome and by whether the frog was euthanased or found dead, from which* Aeromonas hydrophila *was isolated on routine culture*

	GIT	tongue	spleen	heart	lung	liver	kidney	muscle	skin	skin ulcer	urinary bladder	oviduct	fat body
R*	20 (5)	33 (3)	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	0 (4)	25 (4)	NE	NE	NE	NE
U	25 (4)	33 (3)	NE	NE	0 (1)	0 (3)	0 (1)	0 (3)	0 (2)	33 (3)	NE	NE	NE
U*	36 (14)	57 (14)	0 (12)	0 (10)	12 (8)	0 (10)	0 (7)	36 (14)	64 (11)	14 (7)	NE	0 (1)	NE
U+U*	33 (18)	65 (17)	0 (12)	0 (10)	11 (9)	0 (13)	0 (8)	29 (17)	53 (13)	20 (10)	NE	0 (1)	NE
U+H	14 (7)	14 (7)	17 (6)	33 (6)	NE	NE	NE	29 (7)	43 (7)	NE	NE	0 (2)	NE
H	53 (19)	47 (19)	60 (15)	80 (10)	75 (4)	67 (9)	100 (3)	63 (19)	72 (18)	NE	100 (1)	50 (6)	50 (2)
C	33 (4)	NE	NE	NE	NE	100 (4)	75 (4)	75 (4)	NE	NE	NE	NE	NE

(*b*) *Percentage of frogs sampled, by organ and disease syndrome and by whether the frog was euthanased or found dead, from which no bacteria were isolated on routine culture*

	GIT	tongue	spleen	heart	lung	liver	kidney	muscle	skin	skin ulcer	urinary bladder	oviduct	fat body
R*	0 (5)	33 (3)	100 (5)	40 (5)	80 (5)	100 (5)	40 (5)	25 (4)	75 (4)	NE	NE	NE	NE
U	25 (4)	0 (3)	NE	NE	100 (1)	0 (3)	0 (1)	0 (3)	0 (2)	0 (3)	NE	NE	NE
U*	0 (14)	21 (14)	75 (12)	80 (10)	75 (8)	70 (10)	71 (7)	14 (14)	0 (11)	14 (7)	NE	100 (1)	NE
U+U*	6 (18)	18 (17)	75 (12)	80 (10)	78 (9)	54 (13)	62 (8)	12 (17)	0 (13)	10 (10)	NE	100 (1)	NE
U+H	0 (7)	0 (7)	17 (6)	0 (6)	NE	NE	NE	0 (7)	0 (7)	NE	NE	0 (2)	NE
H	0 (19)	0 (19)	27 (15)	0 (10)	0 (4)	0 (9)	0 (3)	0 (19)	0 (18)	NE	0 (1)	17 (6)	0 (2)
C	0 (4)	NE	NE	NE	NE	0 (4)	0 (4)	0 (4)	NE	NE	NE	NE	NE

Numbers in brackets are the number of frogs sampled for bacteriology

Key: NE = not examined, * = euthanased frogs, R = frogs with skin erythema, U = frogs with skin ulceration, H = frogs with systemic haemorrhages, C = control frogs.

Table 5. (*a*) *Percentage of frogs sampled, by disease syndrome and by whether the frog was euthanased or found dead, from which specific bacteria were isolated on routine culture*

	A. hydr.	*A. sobria*	*A. spp.*	*P. putre.*	*P. spp.*	*Cit. freundii*	*Acinet. sp.*	*Flav. breve*	*Flav. spp.*	*Esch. coli*
R*	40	20	60	0	60	20	0	0	0	20
U	60	0	80	0	60	20	40	0	60	20
U*	79	14	86	29	57	36	64	21	43	43
U+U*	74	11	84	21	58	32	58	16	47	37
U+H	57	57	86	57	57	43	29	71	71	29
H	90	25	95	20	55	30	30	55	70	10
C	100	0	100	0	75	0	0	0	0	0

(*b*) *Percentage of tissues sampled from carcasses, by disease syndrome and by whether the frog was euthanased or found dead, from which specific bacteria were isolated on routine culture*

	A. hydr.	*A. sobria*	*A. spp.*	*P. putre.*	*P. spp.*	*Cit. freundii*	*Acinet. spp.*	*Flav. breve*	*Flav. spp.*	*Esch. coli*	no growth
R*	7	2	10	0	10	2	0	0	0	2	56
U	25	0	25	0	20	15	15	0	45	5	10
U*	25	3	29	6	17	11	16	6	12	7	39
U+U*	25	2	28	5	17	12	16	5	17	7	34
U+H	24	40	57	19	19	7	21	24	24	7	2
H	62	13	75	6	22	7	10	24	38	2	4
C	73	0	73	0	40	0	0	0	0	0	0

Key: * = euthanased frogs, R = frogs with skin erythema, U = frogs with skin ulceration, H = frogs with systemic haemorrhages, C = control frogs.

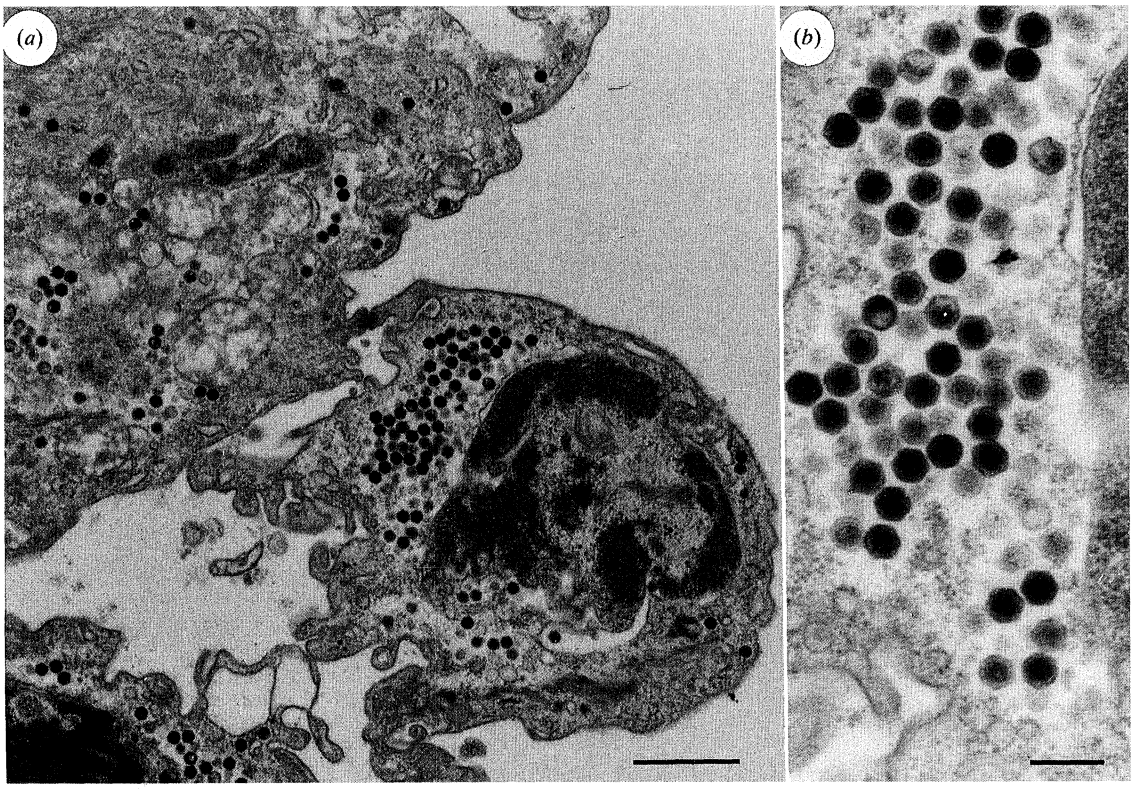

Figure 9. (*a*) Transmission electron micrograph of glutaraldehyde-fixed skin taken from the edge of an ulcer from a frog (ref. 436/92) with ulcerative syndrome. A rounded-up epidermal cell is sloughing from the skin surface. The cell contains a large number of iridovirus-like particles within its cytoplasm, as do the neighbouring epidermal cells. Scale Bar = 1 μm. (*b*) Higher power view of the iridovirus-like particles. Scale Bar = 250 nm.

Aeromonas hydrophila was cultured from one or more of these organs from 16 of 27 (59%) frogs with internal haemorrhages, but from only one of 28 (4%) diseased frogs without this lesion. These results compare with the isolation of *A. hydrophila* from one or more non-GI visceral organs of 16 of 32 (50%) diseased frogs found

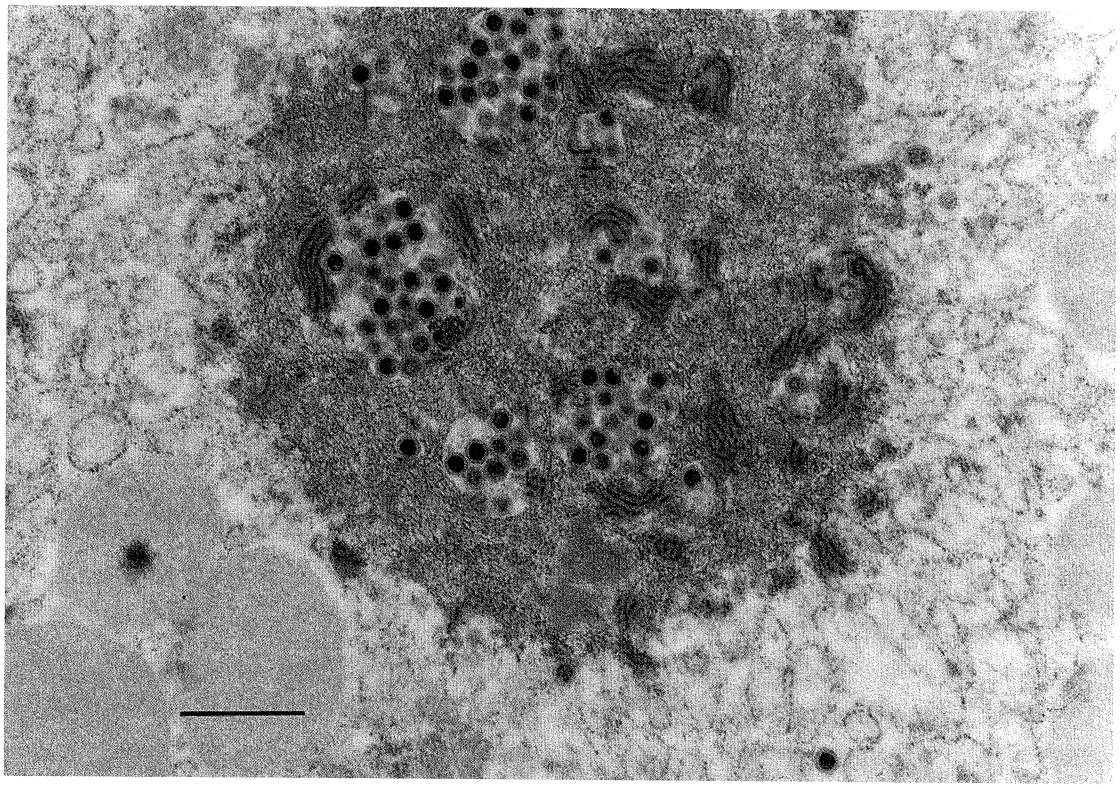

Figure 10. Transmission electron micrograph of a basophilic inclusion body in the liver of a frog (ref. 527/92) with haemorrhagic syndrome. The inclusion is comprised of aggregations of iridovirus-like particles and smooth membraneous structures which may be virus envelope membrane. Scale Bar = 500 nm.

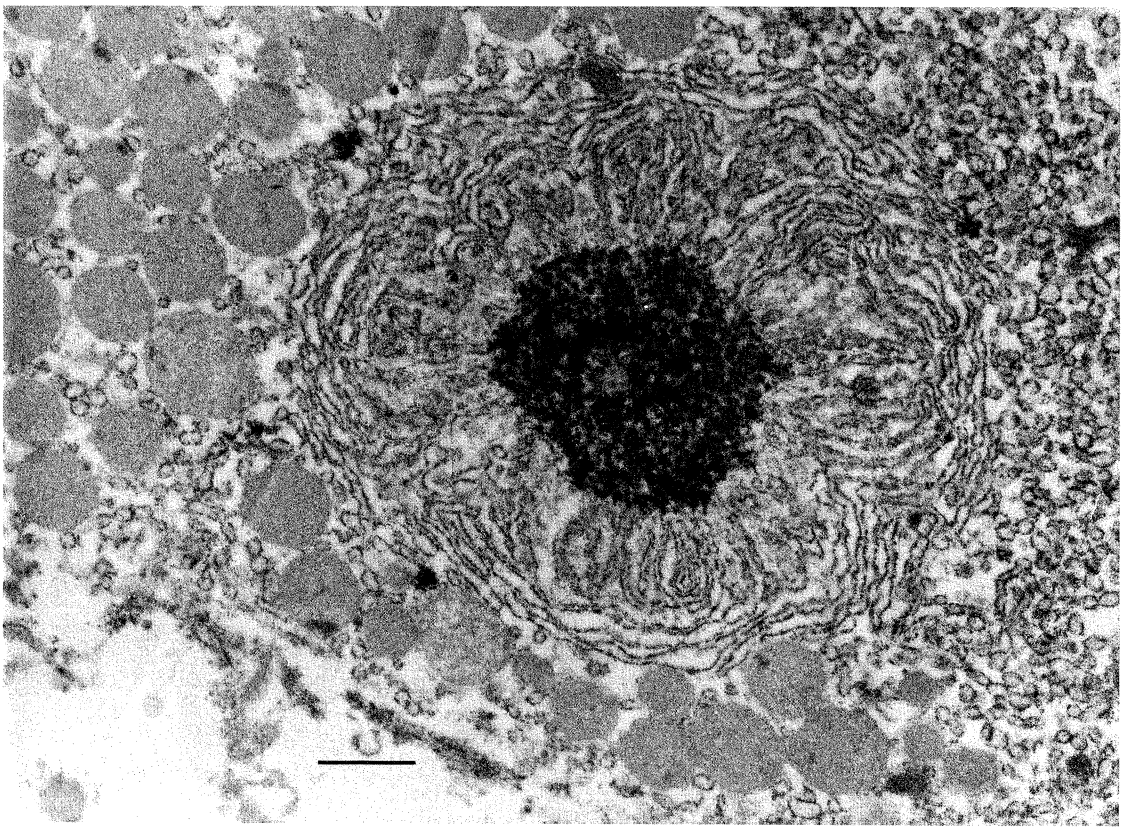

Figure 11. Transmission electron micrograph of a basophilic inclusion body in the liver of a frog (ref. 527/92) with haemorrhagic syndrome. The structure comprises a central core of iridovirus-like particles surrounded by rough endoplasmic reticulum. Scale Bar = 1μm.

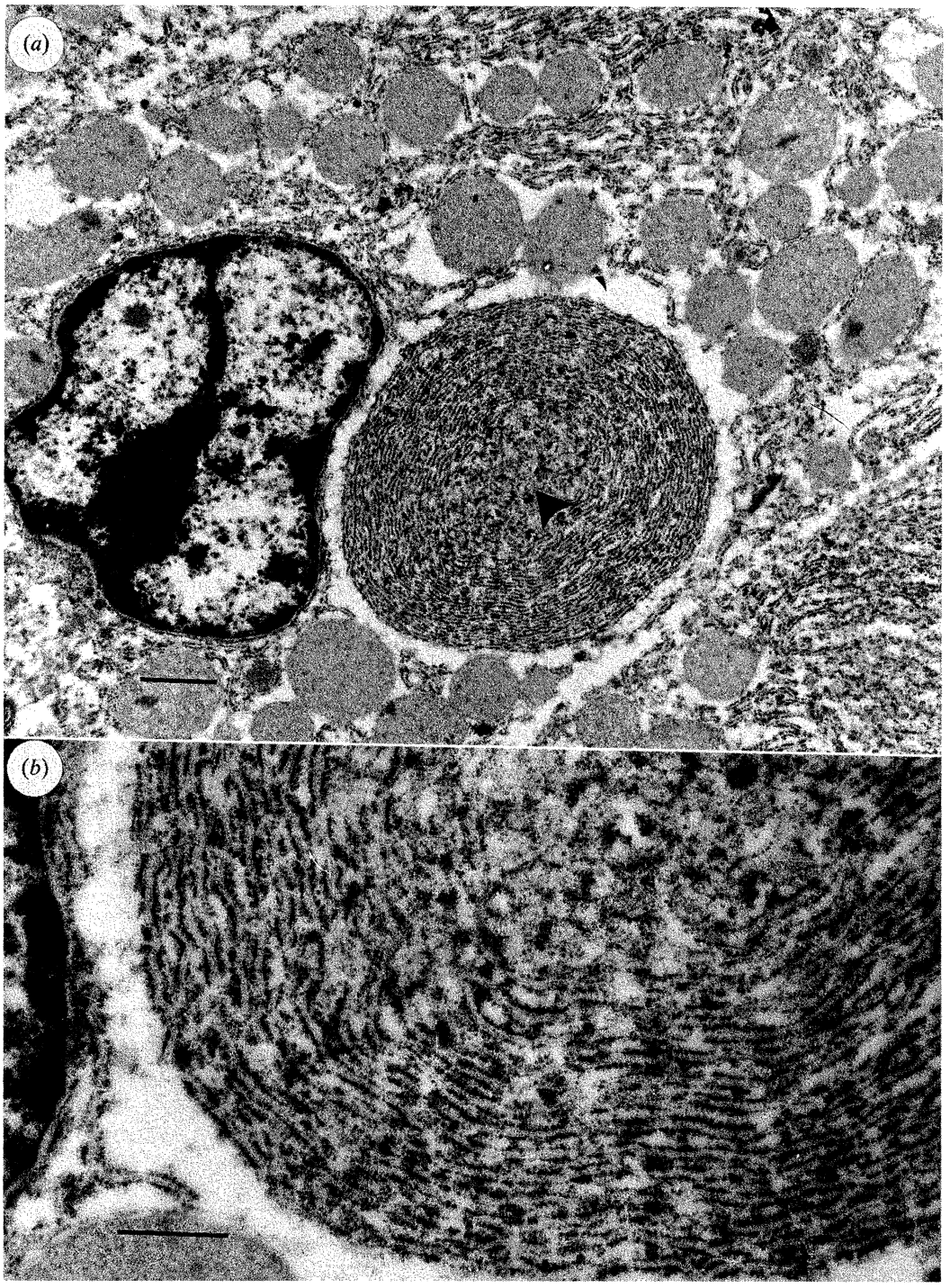

Figure 12. (*a*) Transmission electron micrograph of an acidophilic inclusion body in the liver of a frog (ref. 458/92) with haemorrhagic syndrome. The structure consists of a tight whorl of rough endoplasmic reticulum around an undefined central core (arrowhead). Scale Bar = 1 μm. (*b*) Higher power view of the inclusion showing the rough structure of the endoplasmic reticulum. Scale Bar = 500 nm.

dead and of all four control frogs sampled, but of only one of 19 (5%) euthanased frogs. There was no growth from 10% of these organs sampled from diseased frogs found dead and from none sampled from control frogs, but 74% of the non-GI viscera sampled from euthanased frogs were bacteriologically sterile.

Aeromonas hydrophila was isolated from 20 of 26 frogs with skin erythema, of which 11 had this organism in one or more of their non-GI viscera, and from 22 of 29 frogs without this lesion, including 10 animals from which the organism was isolated from the non-GI viscera. Frogs with RS gave the lowest recovery rate of *A. hydrophila* or related bacteria when compared with frogs with the other disease syndromes and with control animals (tables 4 & 5). Also, frogs with RS had no evidence of septicaemia on bacterial culture, with 72% of the non-GI viscera, including all of the spleens, sampled from these animals being bacteriologically sterile (tables 4 & 5). The only bacteriological difference between frogs with RS and frogs without RS

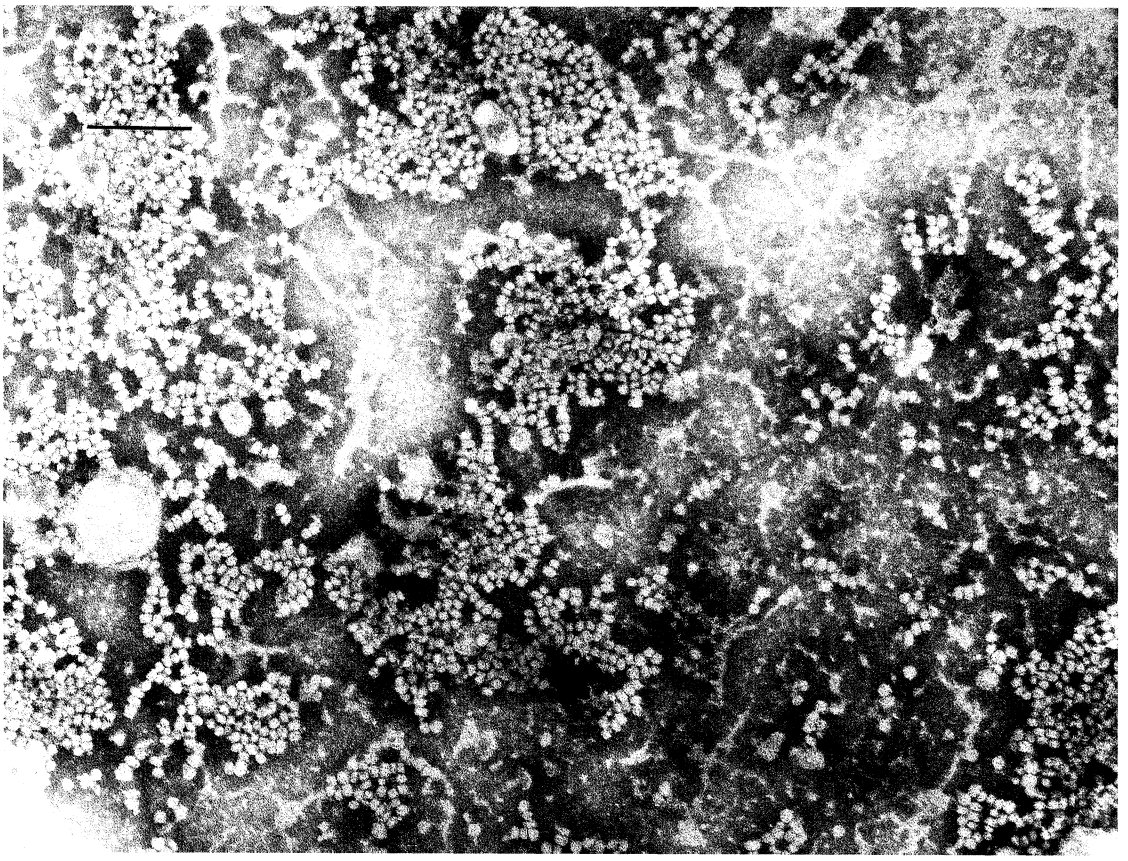

Figure 13. Direct negative contrast electron micrograph of a frog fibroblast cell culture containing a cytopathic effect following inoculation with femoral skin from a frog (ref. 449/92) with haemorrhagic syndrome. Note numerous small round virus-like particles. Scale Bar = 200 nm.

was the isolation of *Corynebacterium sp.* from the alimentary tract and viscera of three of five frogs with RS. This genus of bacterium was not found in any of the other diseased frogs examined in this study, but was cultured from a kidney from one of the control frogs.

No significant anaerobic organisms were grown and, of the samples plated onto Sabouraud's agar, all were negative for fungi except for one (erythematous skin from frog 460/92) from which a mixed growth of mucoraceous moulds was cultured. No acid-fast organisms were found on scrutiny of smears stained with Ziehl-Neelsen's stain.

(*d*) *Virological examinations*

Results of examinations of skin samples from the diseased frogs using negative contrast EM have been presented previously (Cunningham *et al.* 1993). In summary, poxvirus-like particles were found in samples of femoral skin from 48 of 50 frogs examined using this method. In the current study, similar poxvirus-like particles were seen in the femoral skin from five of six control frogs examined using this technique. On TEM examination, the particles failed to show typical poxvirus morphology, but revealed an internal structure characteristic of melanosomes (Ghadially 1980; Fawcett 1981).

Iridovirus-like particles (mean size of ten particles, measured from face to face, was 142 nm, range 136 to 145 nm) (figure 9) were detected in four of seven glutaraldehyde-fixed skin samples examined using TEM (table 2). Epidermal cells containing these particles were seen to be 'rounding up' and sometimes were apparently sloughing from the skin surface (figure 9). Additionally, iridovirus-like particles were seen in two (449/92 & 460/92) of three de-waxed skin samples examined. In both samples, particles were present in areas of epidermis found on histological examination to contain necrosis and in one (449/92), the tissue structure was preserved well enough to distinguish between healthy areas of epidermis, in which no particles were seen, and necrotic areas, in which particles were visible. Similar iridovirus-like particles were seen in the sample of spleen examined using TEM. Most of the particles appeared to be extracellular, scattered throughout necrotic areas, but some intracytoplasmic aggregations also were seen, apparently within lymphocytes or macrophages.

On TEM examination of liver samples taken from paraffin wax blocks, basophilic inclusions detected histologically were shown to be comprised of aggregations of iridovirus-like particles (mean size of ten particles, measured from face to face, was 129 nm, range 120 to 133 nm) (figure 10). Some of these aggregations were surrounded by layers of rough endoplasmic reticulum (RER) (figure 11). The acidophilic inclusions were found to comprise whorls of RER surrounding undefined central cores (figure 12).

A CPE, characterized by foci of rounded-up cells, was seen in six inoculated cell cultures. This CPE was

seen on first passage only of inoculum from one frog, on both first and second passage of inocula from four frogs, and on second passage only of inoculum from one frog. On examination of the affected cells using EM, small, round virus-like particles of variable size (mean 20 nm) and little surface structure were seen (figure 13). Similar particles were detected in smaller numbers in control cultures of the same commercially available cell line which had not been inoculated with material from diseased frogs. An adenovirus-like particle was detected in one of the inoculated cell cultures with a CPE, in addition to the small, round particles.

4. DISCUSSION

Examinations of frog carcasses collected from sites of unusual mortality revealed two main disease syndromes: animals were noted either as having skin ulceration with or without necrosis of distal limbs, or as having systemic haemorrhages, most notably involving the skeletal muscle and the alimentary and reproductive tracts. At three sites of mortality investigated, frogs were found with a combination of skin ulcers and systemic haemorrhages, while at six sites the ulcerative and haemorrhagic syndromes were mutually exclusive to the carcasses examined and usually one of the syndromes predominated at each site investigated (table 2). At one site (site 1) five frogs necropsied had reddened skin only (one of which had focal intramuscular haemorrhages), and one frog had skin erythema and ulceration. Approximately 40 frogs had been found dead with reddened skin and skin ulcers at this site the previous summer, therefore the frogs collected may have been recovering from, or partially refractory to, the ulcerative disease.

All 29 frogs examined with systemic haemorrhages (HS and U + HS) were found dead, while 14 of the 19 frogs examined with skin ulceration only were euthanased. This may reflect the visibility of the lesions, but it also may be a function of the time course of the respective diseases: At sites where frogs were dying with HS, sick frogs were not apparent at the time the affected pond was visited, although there were reports of such frogs being found *in extremis*, often bleeding from the mouth and anus (Langton & Cunningham, unpublished observations). At ponds visited where frogs were dying with US, often diseased frogs, including animals with extensive necrosis of one or more distal limbs, were seen alive in addition to those found dead. Also, frogs with US generally were in poorer bodily condition when compared with frogs with HS, suggesting a longer time course of the former disease syndrome.

The epidermis of the adult common frog is reported to be comprised of five or six layers of cells (Fox 1977), which may vary according to the region of the body (Fox 1974). On histological examination, areas of apparent mild epidermal thickening were seen in the femoral skin of seven of the diseased frogs examined, but also in two of six control animals. In 26 diseased frogs, foci of markedly thickened epidermis were seen with the formation of plaque-like and papilliform structures. It is difficult to say, however, how much of the epidermal thickening was true hyperplasia. Large areas of epidermal necrosis, often with separation from the dermis, were seen in 17 of 19 frogs with systemic haemorrhages, but not in frogs without this lesion. This suggests that the epidermal separation is a real lesion and not due to sectioning artefact. The epidermis was not present on the histological sections from nine frogs, eight of which had systemic haemorrhages. In at least some of these cases, this loss may have been a consequence of epidermal necrosis.

Three of five spleens in which white foci were seen on gross examination were examined histologically and the lesions were found to be of acute necrosis. In addition, necrotic foci which were not apparent grossly were seen microscopically in nine frogs. Since 19 spleens were not examined histologically, the incidence of splenic necrosis may be higher than that detected so far.

Renal myelopoietic tissue was present in all frogs examined histologically in this study, including four control frogs. Necrosis of this tissue was found in all of the frogs with systemic haemorrhages examined histologically, but in only two of the diseased frogs similarly examined without this lesion and in none of the control frogs examined. The systemic haemorrhages and the myeloid necrosis may share a common aetiology, however, it is also possible that the latter lesion predisposed frogs to infection with secondary pathogens which in turn caused the haemorrhages. The apparent relative lack of septicaemia in the frogs with skin ulceration only (tables 4*b* & 5*b*) may support this hypothesis, but further studies are required in order to ascertain the pathogenesis of the lesions seen.

Incidents of frog mortality, as of other species, occur for various reasons. However, a combination of epidemiological, pathological and electron microscopical results suggests that the unusual mortalities of common frogs described in this paper may share a common aetiology. Only four sites were exclusive to one disease syndrome and, although different pathological entities appear to have been found, there was overlap of lesions between the disease syndromes described in this study. Therefore, the demarcation of frogs into separate disease categories may be false when considering the aetiology of the lesions, as the syndromes seen may be different manifestations of the same disease.

One explanation for the occurence of different disease syndromes with a common aetiology would be if frogs with systemic haemorrhages had died during an earlier phase of the disease than frogs without these lesions. There was histological evidence of the formation of epidermal ulceration in frogs with HS while frogs with U + HS generally had areas of apparent epidermal ulceration surrounding small dermal ulcers and frogs with US had relatively large areas of dermal ulceration. These findings may suggest a chronological progression of the skin lesions. The degree of challenge, or the presence or absence of cofactors may be important for the pathogenesis of the syndromes decribed in this study: for example, invasion of bacteria, such as *A. hydrophila*, through early skin lesions (epidermal necrosis) may result in septicaemia

and death before the formation of dermal ulceration. Another possible factor determining the type of disease syndrome which develops may be temperature: for example, the pathogenicity of the rhabdovirus which causes spring viraemia of carp is temperature dependant (Fijan 1988). Alternatively, it is possible that there are two different primary pathogens present, with one causing the ulcerative syndrome and another causing the haemorrhagic syndrome. Although there was no clear evidence for this in the current study, a number of potential candidate aetiological agents were found and these, along with the likelihood of their involvement in the above disease syndromes, will now be discussed.

(*a*) *Candidate aetiological agents*

(*i*) *Aeromonas hydrophila*

The lesions described for each of the disease syndromes in the present study could be consistent with a diagnosis of 'red-leg' using criteria published previously (see above). A large number of species of bacteria has been recorded as causing 'red-leg' (Glorioso *et al.* 1974; Anver & Pond 1984) with *A. hydrophila* being the most frequently reported of these. However, often bacteriology was, or may have been, performed on frogs found dead, bringing the causal link between the bacteria isolated and the disease seen into question (Glorioso *et al.* 1974). 'Red-leg' has been reproduced experimentally by inoculating frogs with *A. hydrophila* and with a number of other bacteria, but the significance of these results to natural disease is unclear (Glorioso *et al.* 1974).

Aeromonas hydrophila often was cultured from the skin (57% of diseased frogs) and alimentary tract (57%) with no differences in the frequency of isolation from these tissues between frogs found dead and frogs euthanased, or between disease syndromes. Also, this bacterium commonly was isolated from control frogs. These findings probably reflect the widespread distribution of *A. hydrophila* in fresh water habitats (Glorioso *et al.* 1974; Nyman 1986) and its commensal nature in the amphibian gut (Hird *et al.* 1981). However, despite isolation of the organism from the skin and gut of frogs with US, and despite areas of extensive dermal ulceration, *A. hydrophila* was not isolated from the heart, liver, kidney or spleen from any frogs with US, and often the non-GI viscera from these frogs were bacteriologically sterile (table 4).

Although differences were found between the bacterial flora of frogs with different syndromes, these must be interpreted with caution because, whilst all of the frogs examined with systemic haemorrhages were found dead, 19 of 24 diseased frogs without this lesion were euthanased. For example, there appears to be an association between the presence of *A. hydrophila* in the non-GI viscera (suggesting septicaemia) and the presence of systemic haemorrhages, however the former may equally be a factor of post mortem bacterial invasion of tissues. These associations can be tested statistically because, as *A. hydrophila* was found in frogs from all incidents of mortality investigated, including the control animals, all of the animals necropsied potentially were exposed to this organism. Of the 55 frogs necropsied and sampled for bacteria, *A. hydrophila* was isolated from at least one non-GI visceral organ from 14 of 20 frogs with systemic haemorrhages and from five of 28 frogs without this lesion. Using the chi-squared test for contingency (Kirkwood 1988), the association between the presence of *A. hydrophila* in the non-GI viscera and the presence of systemic haemorrhages is significant ($\chi^2_1 = 9.1, P < 0.01$). Also, there is a significant association between the presence of *A. hydrophila* in the non-GI viscera and the presence of post mortem invasion (ie. whether frogs were found dead as opposed to having been euthanased) ($\chi^2_1 = 11.4, P < 0.001$). These analyses indicate that *A. hydrophila* is more likely to be isolated from the non-GI viscera of a frog if that animal was found dead than if that animal had systemic haemorrhages. However, the association between this lesion and the presence of *A. hydrophila* is strong and the possibility of the haemorrhages being caused by this organism cannot be ruled out. If the above analysis is conducted on only the 36 frogs found dead, of which 27 had systemic haemorrhages, no association is found between this lesion and the presence of *A. hydrophila* in the non-GI viscera ($\chi^2_1 = 0.6, P < 0.5$), but, as the number of frogs found dead without haemorrhages was small, this result must be treated with caution. Also, although there is a significant association between the absence of *A. hydrophila* in the non-GI viscera and the presence of skin ulcers ($\chi^2_1 = 15.4, P < 0.001$), this may be a factor of the absence of post mortem tissue invasion. Interestingly, in contrast to the findings in the literature previously, there is no association between skin erythema and the isolation of *A. hydrophila*, from either the non-GI viscera only or from all tissues sampled, in the current study.

(*ii*) *Other species of bacteria*

None of the bacteria other than *A. hydrophila* were found in all of the sites investigated, therefore possible associations with observed lesions cannot be analysed statistically. However, although the patterns of isolation of all but one of the other bacteria cultured were similar to that for *A. hydrophila*, i.e. isolated from more animals and more tissues from frogs with systemic haemorrhages/found dead than from frogs with US/euthanased, these were less pronounced (table 5). Only *Acinetobacter sp.* was isolated from frogs with US/euthanased more frequently than from frogs with systemic haemorrhages/found dead and this organism appeared to be associated with areas of skin ulceration.

(*iii*) *Iridovirus-like particle*

An iridovirus-like particle was seen in skin from six of ten diseased frogs examined using TEM, either at the periphery of skin ulcers (frogs with US), or in areas of epidermal necrosis (frogs with HS), but not in adjacent areas of healthy epidermis. Also, a similar particle was found associated with necrotic foci in a sample of spleen examined using TEM. This spleen was from a frog with US and no bacteria were detected in the organ either

on routine bacteriology or using TEM. No iridovirus-like particle was seen on examining skin using direct negative contrast EM. Although this may be because the samples examined using TEM were specifically of skin lesions (ulcers or epidermal hyperplasia), similar lesions were present in many of the femoral skin samples examined histologically and probably were present in the samples examined using EM.

An iridovirus-like particle also was detected using TEM examination of the basophilic inclusions seen in hepatocytes. Although these inclusions were detected only in frogs with systemic haemorrhages, they were apparently not associated directly with hepatocellular necrosis or other lesions. Acidophilic inclusions seen on microscopical examination of the liver were found to comprise whorls of RER on examination using TEM. The aetiology of these inclusions is not known, but some of the iridovirus-like (basophilic) inclusions were seen to be surrounded by layers of RER on examination using TEM and the presence of acidophilic bodies closely mirrored that of basophilic bodies. Therefore, it may be that both types of inclusion are a consequence of infection with the iridovirus-like particle.

Since the above virological examinations were conducted, an iridovirus-like particle, similar to that seen in the current study, has been isolated from ulcerated common frog carcasses collected from further mortality incidents in the south east of England (Drury *et al.* 1995). The particle was cultured from skin and from viscera in three cell lines, including the same frog cell line as that used in the current study.

Iridoviruses, or iridovirus-like particles, have been reported from species of anuran previously (Mişcalencu *et al.* 1981; Granoff 1989; Alves de Matos & Paperna 1993). Focal haemorrhages in the viscera and muscle were seen in toads (*Scaphiopus sp.* & *Bufo spp.*) infected experimentally with Tadpole Edema Virus (TEV) (Wolf *et al.* 1968) and experimental infection with Bohle iridovirus (isolated from diseased ornate burrowing frogs (*Lymnodynastes ornatus*)) causes tissue necrosis, including necrosis of the renal and splenic haematopoietic tissue, and death in frogs (Cullen *et al.* 1995). An iridovirus was isolated from edible frogs (*R. esculenta*) with systemic haemorrhages and skin necrosis (Fijan *et al.* 1991), but transmission experiments using cultured virus failed to reproduce the disease. However, an unknown agent, presumed to be a virus, isolated from sick edible frogs by Kunst & Valpotic (1968) was shown to cause disease on inoculation into frogs and this was termed 'Viral Haemorrhagic Septicaemia of Frogs' (VHSF).

In the current study, it would appear that there is a close association between the presence of an iridovirus-like particle and the presence of tissue necrosis, particularly of the epidermis, but further examinations of tissues from healthy and diseased frogs are required before these results can be evaluated fully. Many of the lesions detected are similar to those found in frogs with VHSF (Kunst & Valpotic 1968), to those caused by experimental infection of toads with TEV (Wolf *et al.* 1968) and to those described in frogs following experimental infection with Bohle iridovirus (Cullen *et al.* 1995). Also, lesions similar to those seen in frogs in the present study, such as skin ulceration, hepatic and splenic necrosis, and necrosis of renal haematopoietic tissue, are found in certain naturally-occurring iridovirus infections of fish (Langdon & Humphrey 1987; Langdon *et al.* 1988).

(iv) Poxvirus-like particle

A poxvirus-like particle was detected in the skin of a high percentage of diseased frogs examined using EM and this structure was considered to be a possible aetiological agent, or co-agent, of the frog mortality decribed (Cunningham *et al.* 1993). However, similar particles have been found since in five of six control frogs killed by cats but which were otherwise healthy. Further examinations using TEM have identified this particle as a melanosome and not a pathogen.

(v) Small, round virus-like particle

On inoculating cultures of a frog cell line with homogenates of femoral skin from 32 frogs, CPE was detected in a small number of cultures both on first and on second passages. This suggests the culture of a pathogenic agent, and a small, round particle was detected on examination of the affected cells using EM. However, the identity of this particle is not known, and, as it was present also in control cell cultures, it seems likely that it is either a contaminant virus or a non-virus particle.

(vi) Adenovirus-like particle

In addition to the small, round particle, an adenovirus-like particle was detected on EM examination of CPE following incubation with inoculum from one frog. Adenoviruses have been reported from amphibians twice previously, with both isolates having been cultured from renal tumours in leopard frogs (Clark *et al.* 1973; Wong & Tweedell 1974). The first isolate was designated frog adenovirus 1 (FAV1) (Clark *et al.* 1973) and it is probable that both isolates are of the same virus (Granoff 1989). FAV1 failed to cause disease in transmission studies (Clarke *et al.* 1973) and this virus appears to have been an incidental finding and since has been designated as an 'orphan virus' (Granoff 1989). The adenovirus-like particle described in the current study was cultured from one frog only and also may be incidental to the lesions observed.

5. CONCLUSIONS

Investigations into unusual frog mortality in Britain have resulted in two main disease syndromes being found: one characterized by skin ulceration and one characterized by systemic haemorrhages. However, there was an overlap of lesions between the disease syndromes and frogs with each syndrome shared common microscopic skin lesions, therefore the syndromes seen may be different manifestations of the same disease. The lesions found could be consistent with one or more previous descriptions of 'red-leg'.

How epidemics of 'red-leg' occur naturally is not known, but this disease generally is regarded as having

a primary bacterial (usually *A. hydrophila*) aetiology (Glorioso *et al.* 1974; Anver & Pond 1984). However, there also has been speculation that the bacteria isolated are post mortem invaders (Glorioso *et al.* 1974), or are secondary pathogens invading the host either via skin or gut lesions (Russell 1898; van der Waaij *et al.* 1974), or following exposure to stress agents (Glorioso *et al.* 1974; Hird *et al.* 1981; Carey 1993). Findings from the current study support the hypothesis that bacterial infection is either secondary or post mortem and suggest a possible mechanism for the pathogenesis of 'red-leg' of either primary iridovirus infection only, or primary iridovirus infection followed by secondary infection with opportunistic bacteria.

It has been hypothesized recently that infectious disease may be contributory to an apparent world-wide decline of amphibian populations (Laurance 1995). The findings of Kunst & Valpotic (1968), Wolf *et al.* (1968), Cullen *et al.* (1995) and of Drury *et al.* (1995), along with those presented in the current paper, suggest that there is an urgent need to investigate the role of infectious disease in general, and of iridoviruses in particular, in this apparent decline.

Further work is being undertaken to culture and characterize the virus-like particles reported here and transmission experiments are planned in order to elucidate the pathogenicity of the candidate aetiological agents described in this article.

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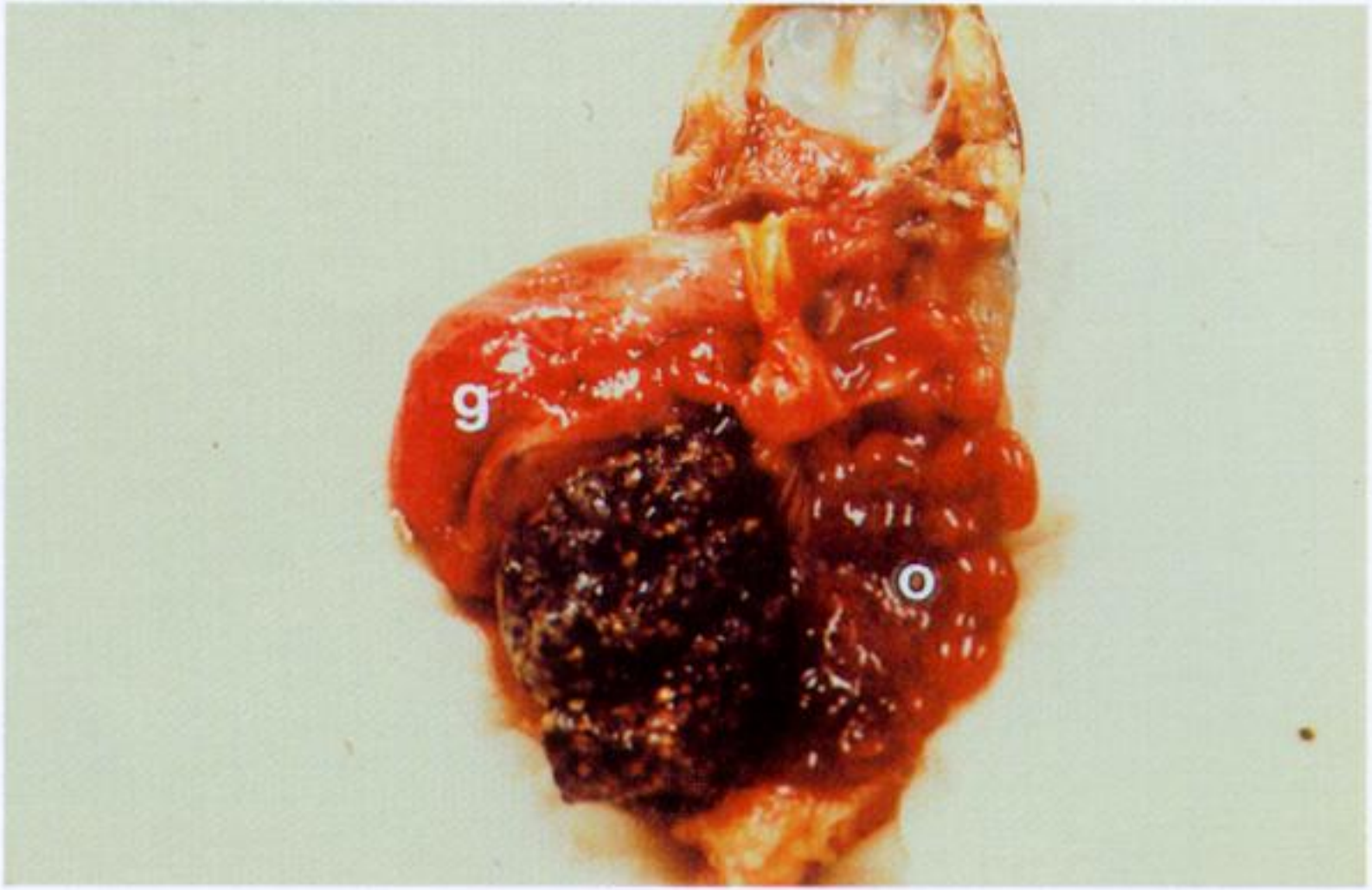

Figure 1. Ventral view of a frog (ref. 485/92) with haemorrhagic syndrome. The ventral body wall and limbs have been removed to reveal the viscera. Note diffuse erythema throughout the viscera, and particularly the haemorrhagic gut (g) and oviduct (o).

Figure 2. Main lesions of frogs with ulcerative syndrome. (a) Ventral view of frog ref. 338/92 with a linear femoral ulcer and severe ulceration of the right forefoot with loss of digits. (b) Frog ref. 339/92 with necrosis of the right hindfoot. (c) Frog ref. 439/92 with extensive skin ulceration of the dorsal aspect of the left thigh.

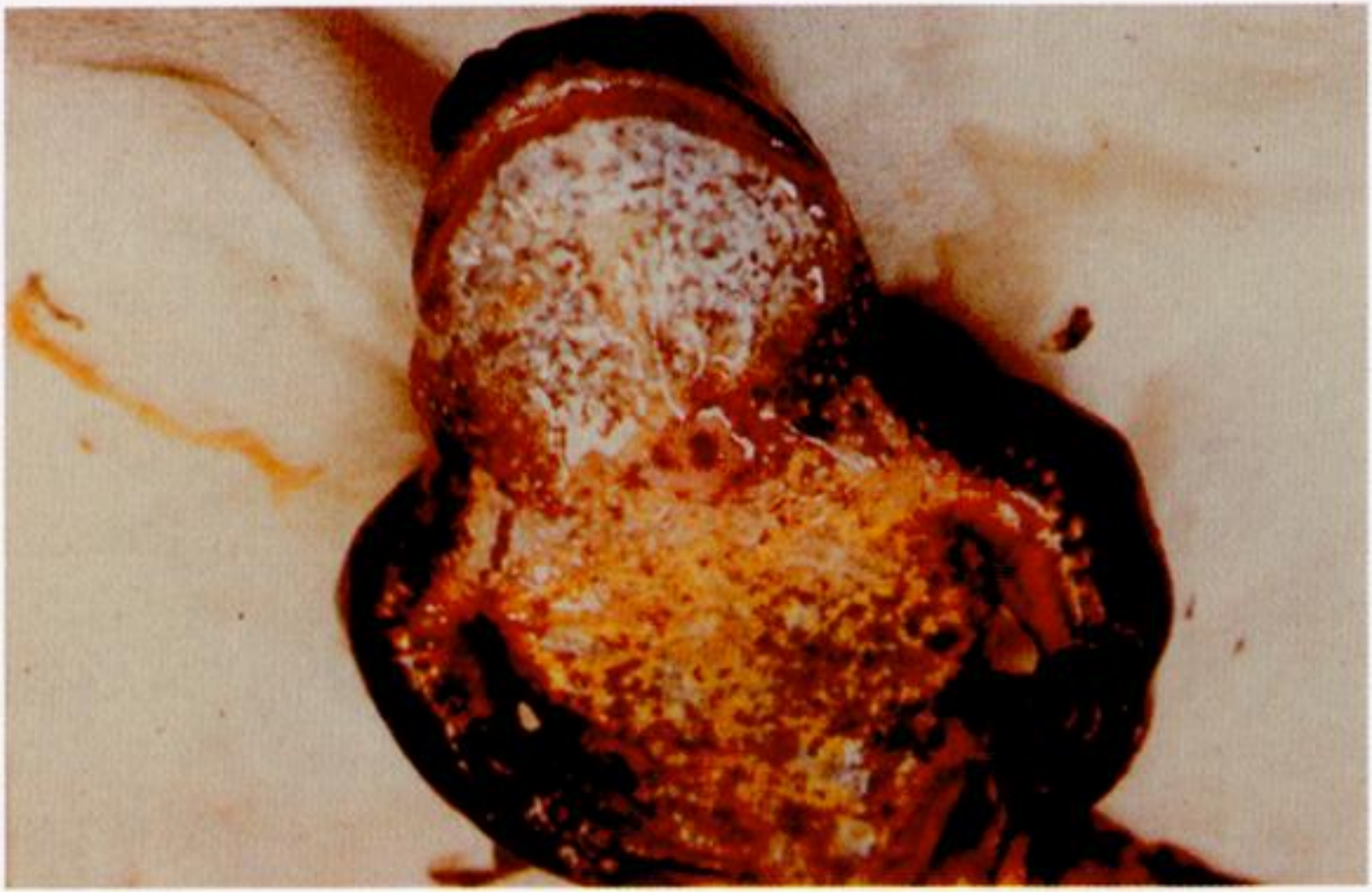

Figure 3. Ventral view of a frog (ref. 464/92) with ulcerative and haemorrhagic syndrome. Note the small central area of dermal ulceration surrounded by a grey zone of epidermal ulceration.

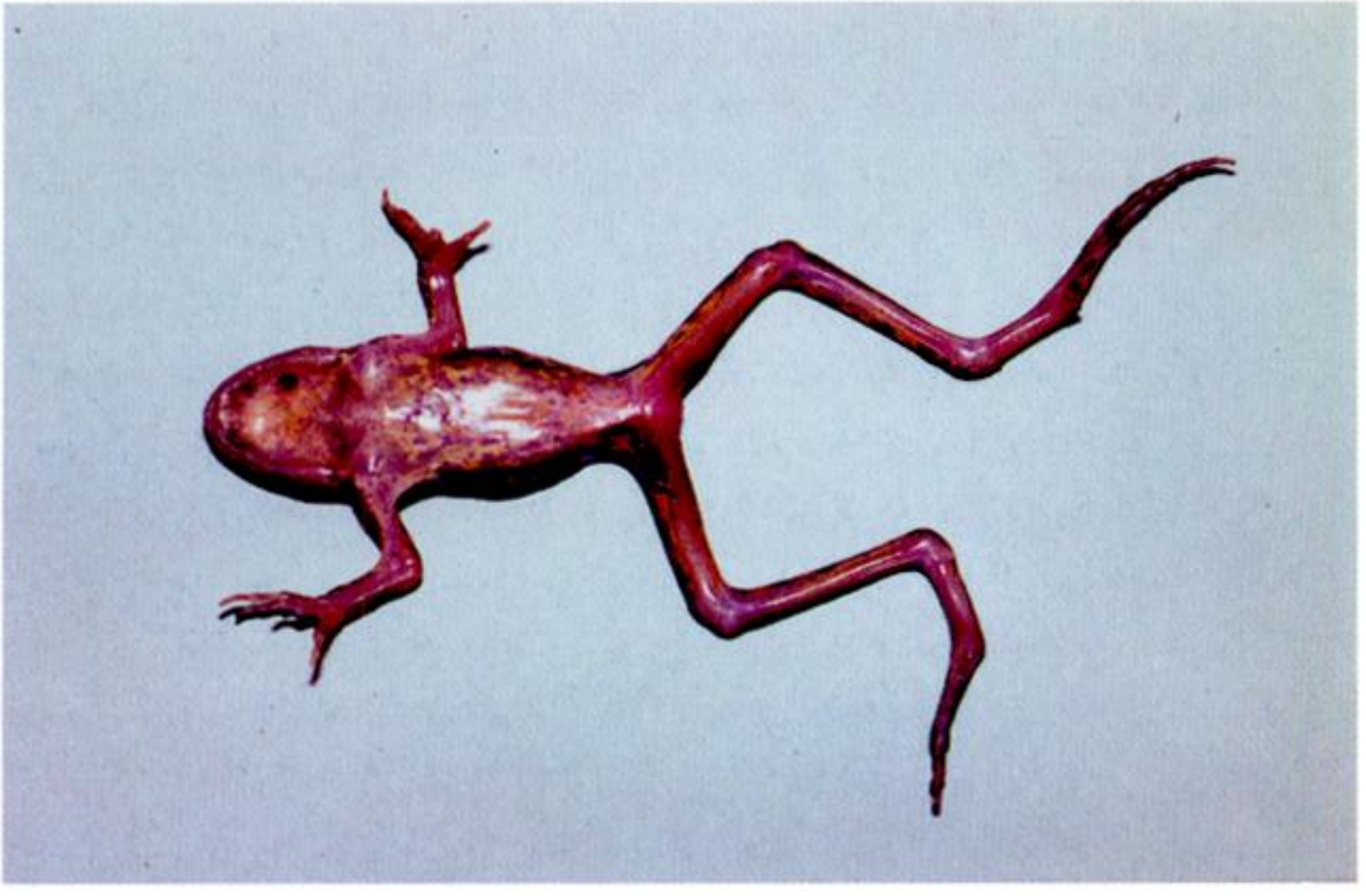

Figure 4. Ventral view of an emaciated frog (ref. 291/92) with ulcerative syndrome. Note the marked wasting of skeletal muscle and skin ulcers over the right thigh, left tarsus and throat.

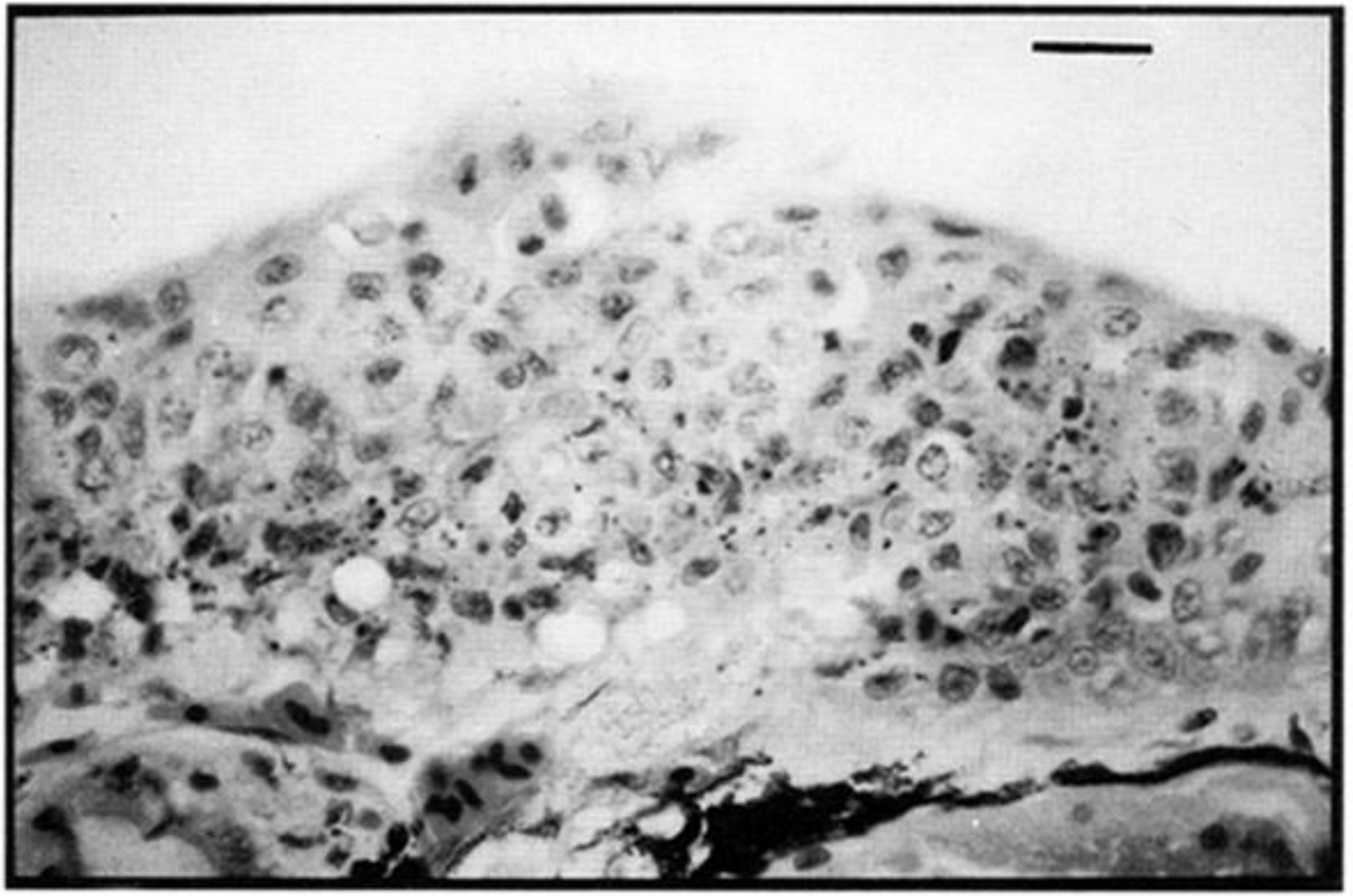

Figure 5. Photomicrograph of femoral skin from a frog (ref. 460/92) with haemorrhagic syndrome showing epidermal thickening with necrosis of the deeper cell layers. H&E. Scale Bar = 25 μm.

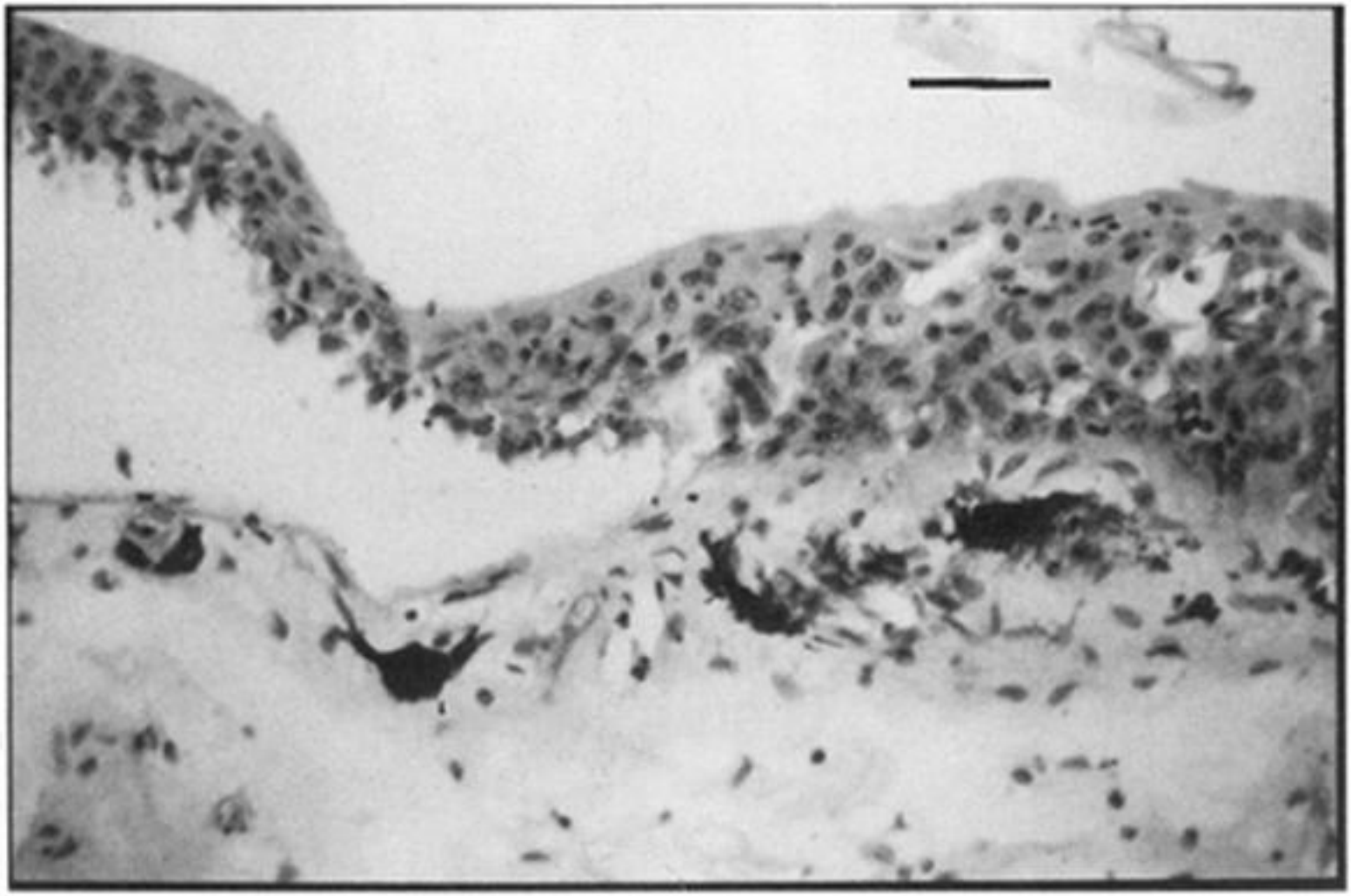

Figure 6. Photomicrograph of femoral skin from the same frog as figure 5 showing separation of the epidermis from the dermis. H&E. Scale Bar = 50 μm.

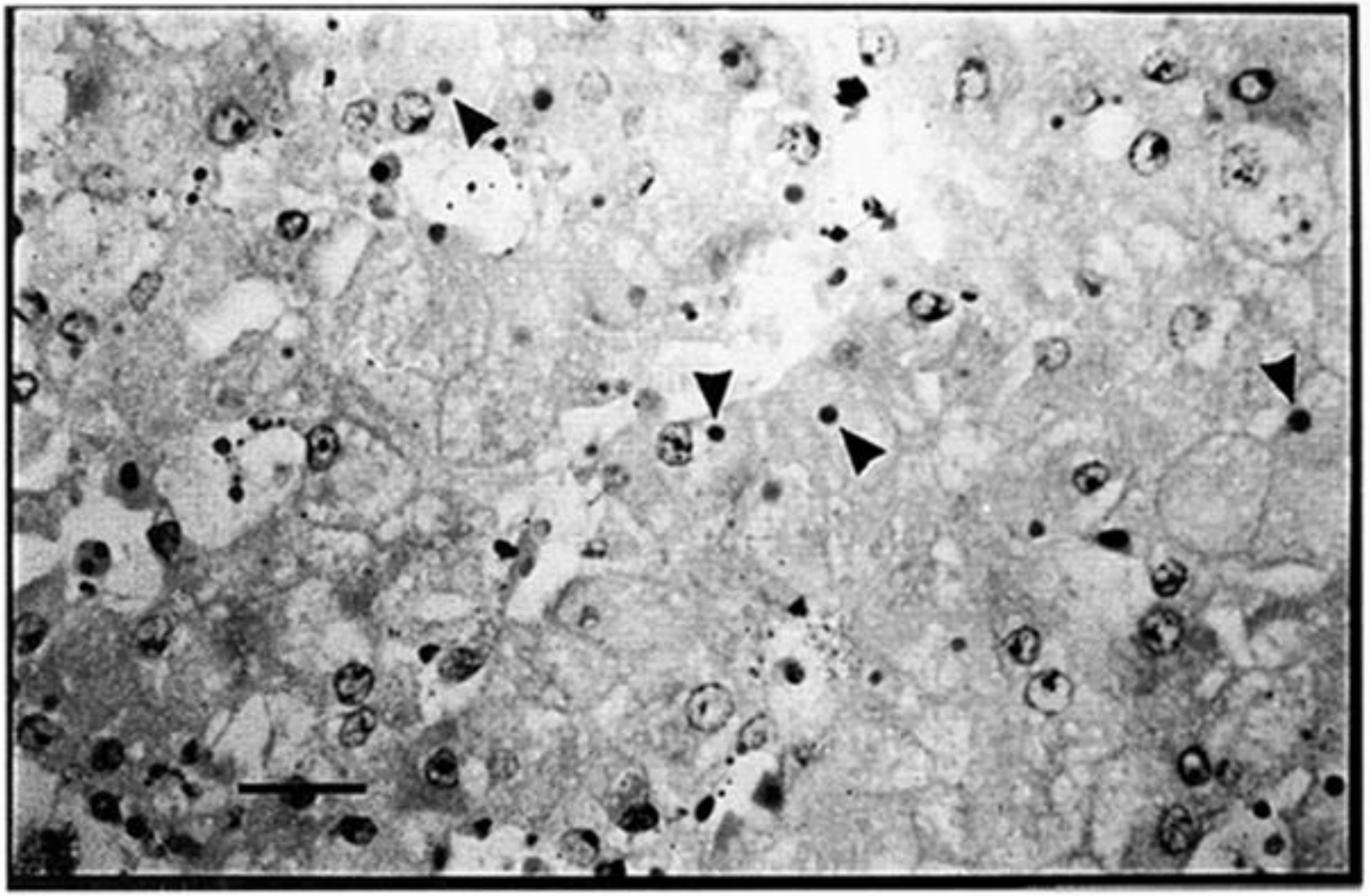

Figure 7. Photomicrograph of liver from a frog (ref. 465/92) with ulcerative and haemorrhagic syndrome showing intracytoplasmic basophilic inclusions (arrowheads) within hepatocytes. H&E. Scale Bar = 25 μm.

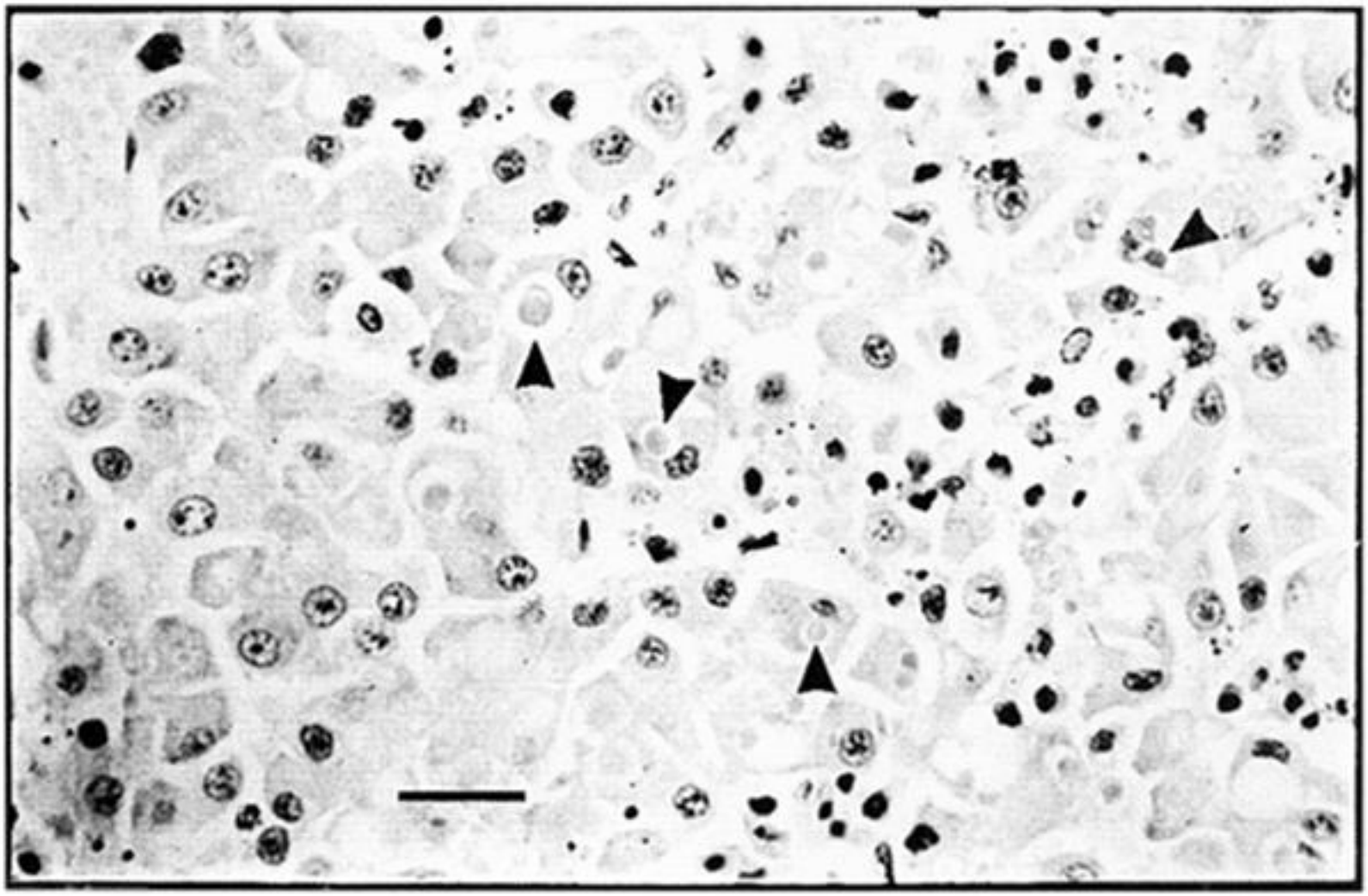

Figure 8. Photomicrograph of liver from a frog (ref. 458/92) with haemorrhagic syndrome showing intracytoplasmic acidophilic inclusions (arrowheads) within hepatocytes. H&E. Scale Bar = 25 μm.

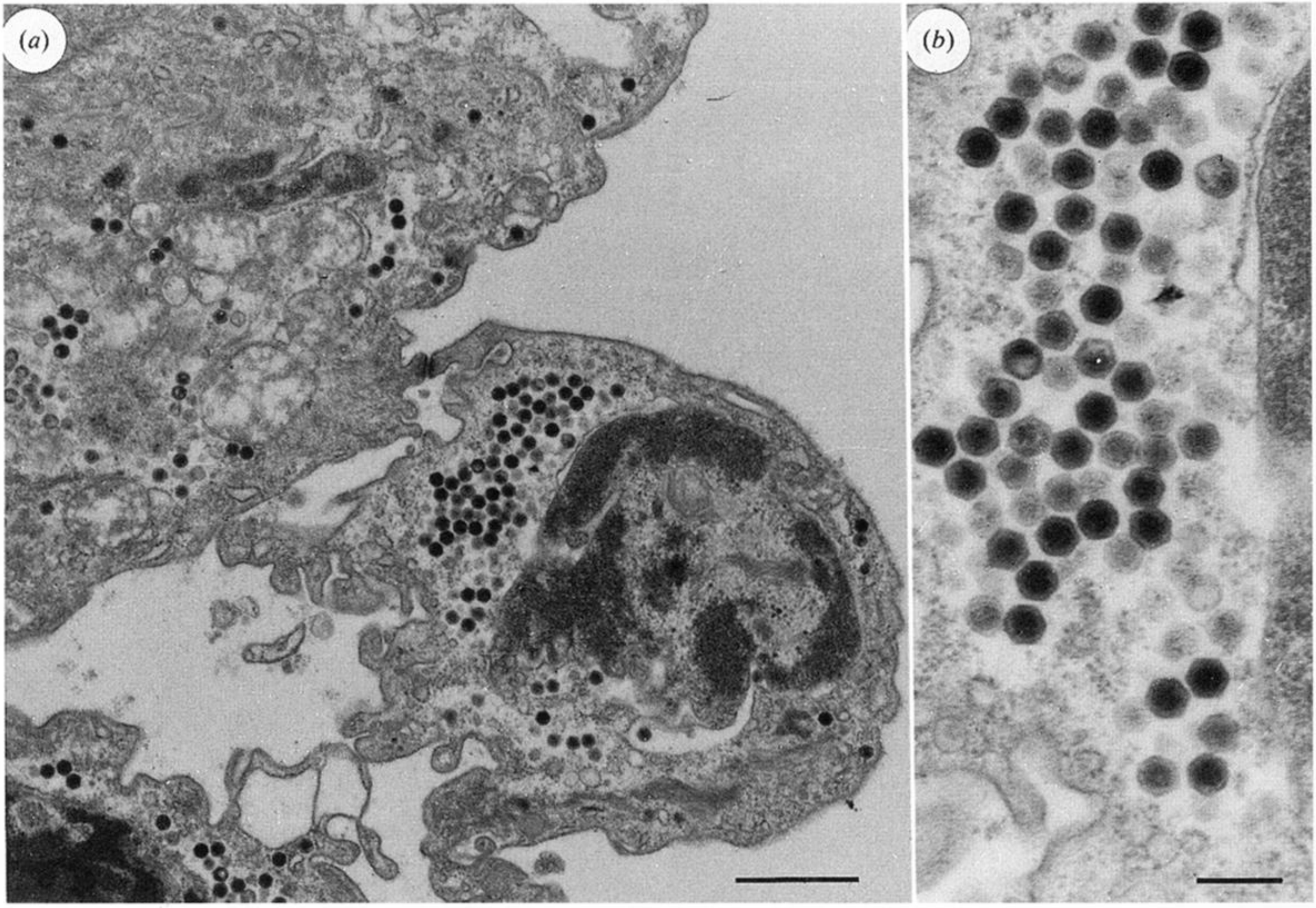

Figure 9. (*a*) Transmission electron micrograph of glutaraldehyde-fixed skin taken from the edge of an ulcer from a frog (ref. 436/92) with ulcerative syndrome. A rounded-up epidermal cell is sloughing from the skin surface. The cell contains a large number of iridovirus-like particles within its cytoplasm, as do the neighbouring epidermal cells. Scale Bar = 1 μm. (*b*) Higher power view of the iridovirus-like particles. Scale Bar = 250 nm.

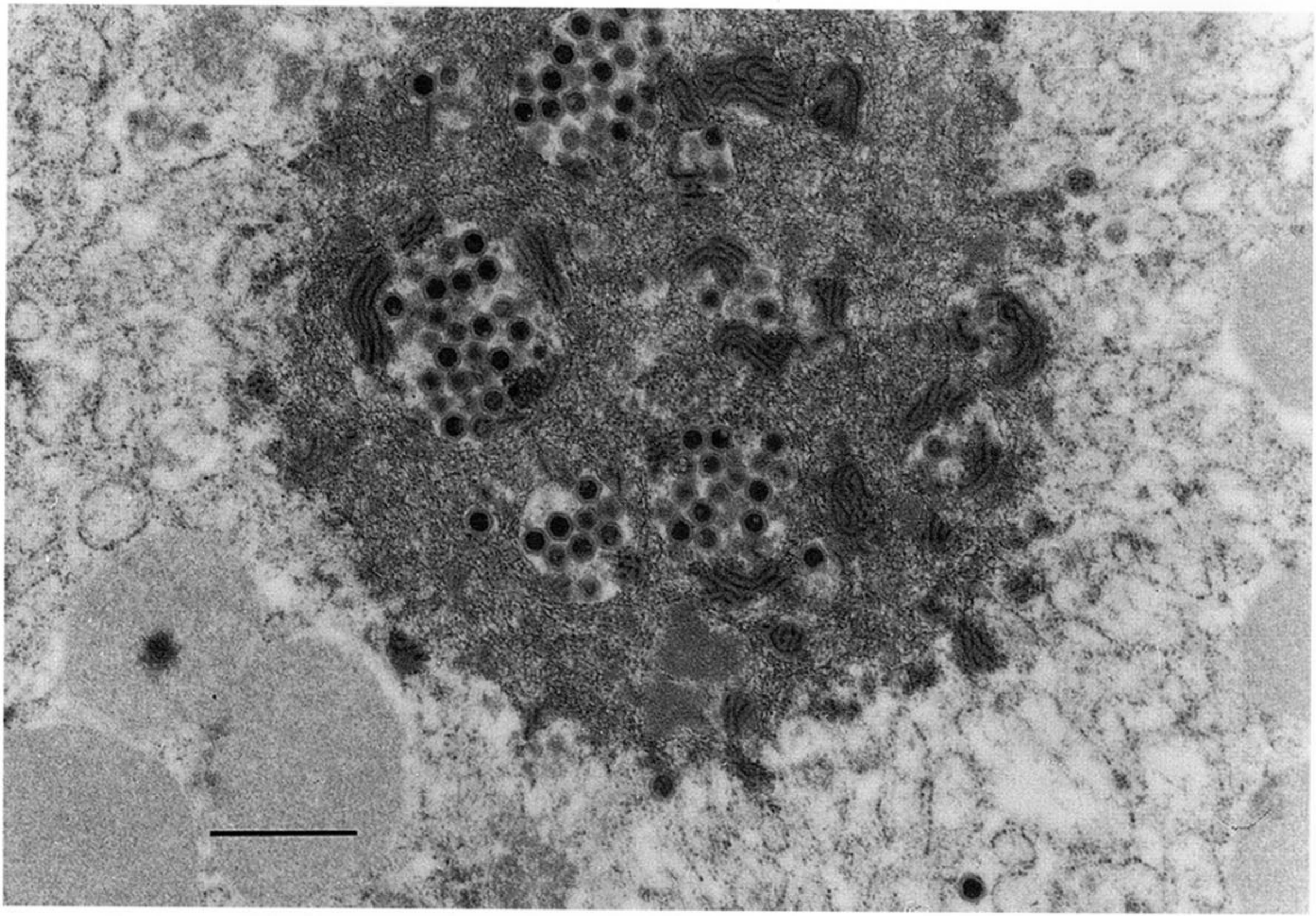

Figure 10. Transmission electron micrograph of a basophilic inclusion body in the liver of a frog (ref. 527/92) with haemorrhagic syndrome. The inclusion is comprised of aggregations of iridovirus-like particles and smooth membraneous structures which may be virus envelope membrane. Scale Bar = 500 nm.

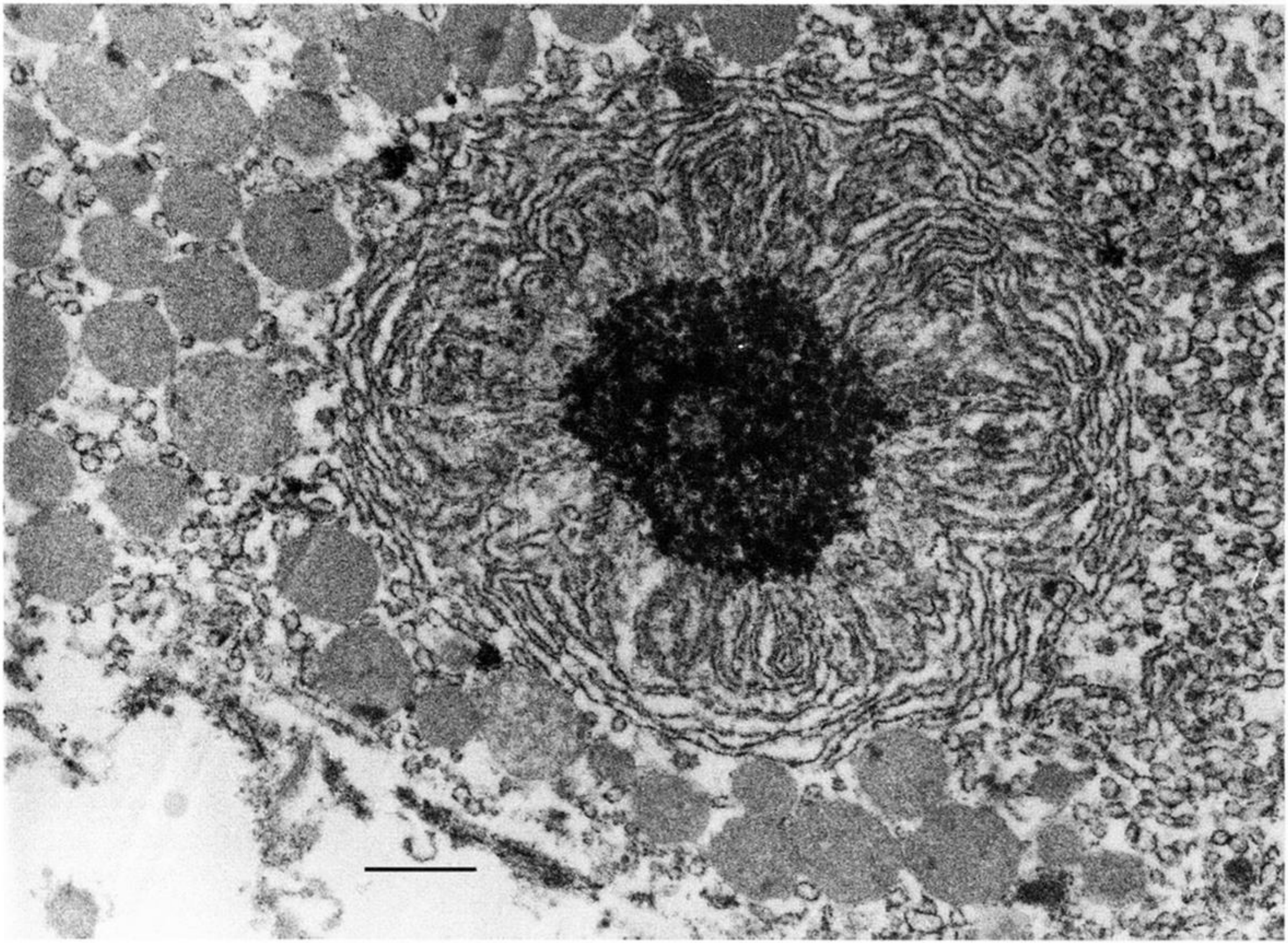

Figure 11. Transmission electron micrograph of a basophilic inclusion body in the liver of a frog (ref. 527/92) with haemorrhagic syndrome. The structure comprises a central core of iridovirus-like particles surrounded by rough endoplasmic reticulum. Scale Bar = 1μm.

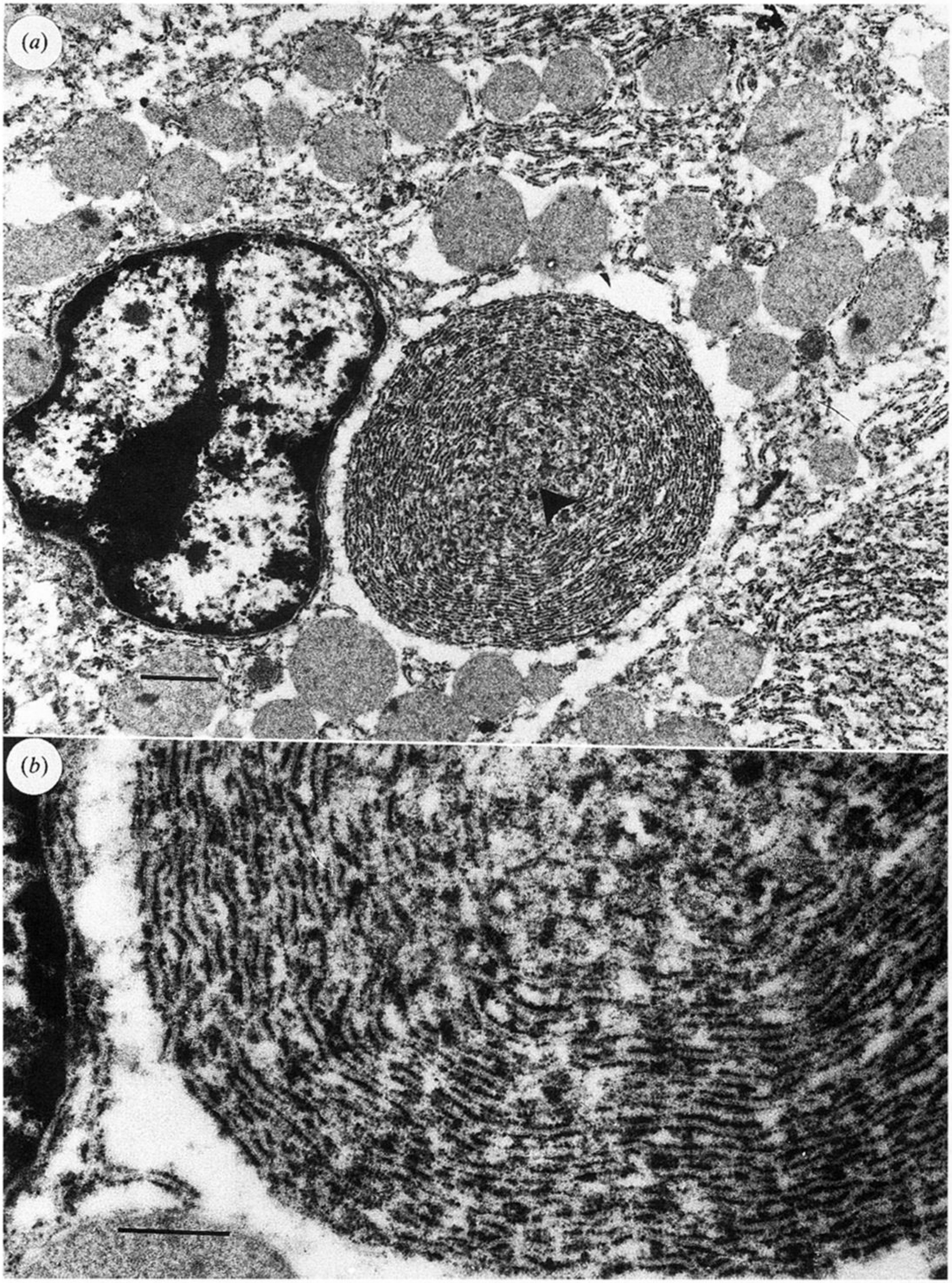

Figure 12. (*a*) Transmission electron micrograph of an acidophilic inclusion body in the liver of a frog (ref. 458/92) with haemorrhagic syndrome. The structure consists of a tight whorl of rough endoplasmic reticulum around an undefined central core (arrowhead). Scale Bar = 1 μm. (*b*) Higher power view of the inclusion showing the rough structure of the endoplasmic reticulum. Scale Bar = 500 nm.

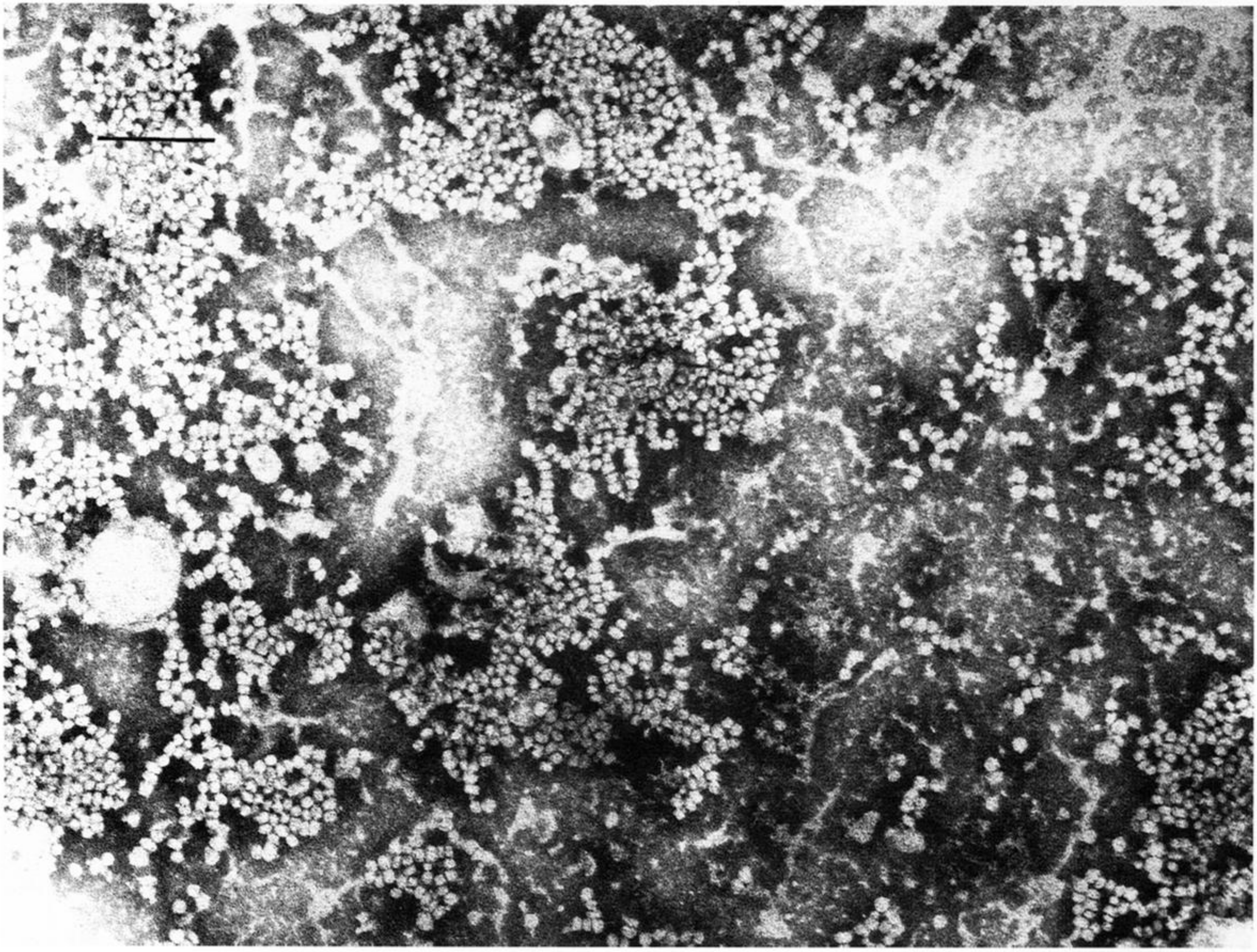

Figure 13. Direct negative contrast electron micrograph of a frog fibroblast cell culture containing a cytopathic effect following inoculation with femoral skin from a frog (ref. 449/92) with haemorrhagic syndrome. Note numerous small round virus-like particles. Scale Bar = 200 nm.