GC analysis on columns A and B and mass spectral data of this peak were also identical. The pseudoionone isomers used as starting materials were inseparable by GC, even with capillary columns, but could be separated using silica gel HPLC with 50% $CHCl_3$ in pentane as solvent. Since Zakharova et al. has previously determined the configuration of the pseudoionone isomers[14b], the possible products of the Wittig reactions were also determined. The active material was found to have the *Z,Z,Z*-configuration.

The pheromone was found to be identical spectrally and chromatographically with *Z,Z,Z*-allofarnesene, but all isomers with *Z*-4 configuration were biologically active. Testing using serial dilutions showed the syntetic *Z,Z,Z* isomer to have noticeable activity at the 5 fg/cm level, but optimum activity was found at the 100–500 pg/cm level (fig.). GC analysis of the pheromone deposited in a 10-cm glass tube by a colony of actively moving ants was also within this range. Ants reaching a section of trail of higher or lower concentration than the one they were moving along hesitated before proceeding, regardless of whether the trail they had been following was natural or artifical. This indicated that the ants could detect differences in pheromone concentration, a useful ability since trails must cross in the field.

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Effect of exogenous iron on the viability of pathogenic *Naegleria fowleri* in serum

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Summary. When *Naegleria fowleri* (Lee) was incubated in newborn calf and human serum an amebicidal effect was observed. Heat inactivation of both sera resulted in the recovery of viable amebae after incubation in these sera. Exogenous iron added to non-heat inactivated calf serum improved viability slightly but was without effect when added to human serum not heat inactivated. Exogenous iron greatly enhanced growth and/or viability in heat inactivated calf serum. Viability of amebae also was considerably enhanced in human serum which was heat inactivated when pH was lowered in conjunction with iron supplements.

Naegleria fowleri is one of several species of free-living amebae which can cause primary amebic meningoencephalitis (PAME). It is usually a fatal disease of the central nervous system, and nearly all cases have been related to recent association with water sports or other aquatic activity[2].

Numerous axenic culture media have been developed for the cultivation of *Naegleria* species[3–8]. Most of these media contain mammalian serum in conjunction with a variety of other components for enhanced *Naegleria* growth.

The growth of amebae in serum-enriched media suggests serum itself might support the growth or maintenance of amebae. It has been shown that mammalian serum could support the growth of some procaryotic parasites provided sufficient iron was initially available or the serum was supplemented with exogenous iron[9, 10].

Mammalian serum has been reported to have an amebicidal or amebostatic effect on amebae[11–15]. These results have been attributed to a variety of factors including antibodies, heat labile factors, and complement. The purpose of this investigation was to quantify the viability and/or growth of pathogenic *N. fowleri* in newborn calf and human serum and to determine if addition of iron could enhance viability and growth.

Materials and methods. Newborn calf serum (Grand Island Biological Co., Grand Island, N.Y.) and pooled human serum (from a local hospital) were heat inactivated at 56 °C for 30 min unless designated otherwise. Penicillin and streptomycin (200 µg/ml each) were added, and the serum was sterilized by filtration (0.45 µm). 2-ml aliquots were pipetted into 15 × 165 mm screw-capped tubes. Iron was added to serum in the form of $FeCl_3 \cdot 6\ H_2O$ or $FeSO_4 \cdot 7\ H_2O$. Stock iron solutions were prepared in distilled water, filter sterilized, and diluted in distilled water so that 0.5 ml delivered the appropriate amount of iron. In addition, the pH of some tubes was lowered with 1 N HCl. Iron and HCl were added to sera 24 h before inoculation with amebae. Control tubes received Page ameba saline (PAS)[16]. *Naegleria fowleri* (Lee) was grown at 37 °C in tubes containing 3 ml of Chang's SCGYEM medium[6]. For inoculum, amebae were harvested by centrifugation (1000 × g for 10 min) and resuspended in PAS. The number of amebae/ml were determined by hemocytometer counts and dilutions were prepared in PAS so that 0.5 ml delivered the

appropriate number of amebae. Tubes containing serum, iron, HCl, and amebae were incubated at 37 °C on a 45° angle.

For quantification and determination of ameba viability after incubation in serum, a plaque technique was utilized[17]. When amebae are mixed with bacteria and inoculated onto inorganic agar plates, individual amebae ingest bacteria and multiply forming a plaque. A 0.1-ml volume was taken from experimental tubes and diluted to 10^{-2} in PAS. Aliquots from each tube were also inoculated into trypticase soy broth (TSB) to check for bacterial contamination. A volume of 0.25 ml was taken from the dilution tube and pipetted onto inorganic agar plates (15 × 100 mm). A pellet of *Escherichia coli* (obtained by centrifugation) from a 72-h TSB culture was then added to agar plates. The pellet and ameba suspension were evenly spread over the agar surface with a bent glass rod. After the fluid was absorbed by the agar, plates were inverted and incubated at 37 °C. After 48 h the number of amebae or plaque forming units (pfu) were counted.

Results. The effect of exogenous iron on ameba viability in newborn calf serum is given in table 1. In heat inactivated serum the addition of 200 µg Fe/ml promoted a marked increase in pfu (table 1). This growth was confirmed also by hemocytometer count. Because addition of $FeCl_3$ lowered the pH of serum to 6.2 an additional control was made utilizing 1 N HCl to lower pH. A considerable amebicidal effect was observed in these acidified tubes. In newborn calf serum not heat inactivated and with no exogenous iron, the presence of an amebicidal factor was apparent (table 1). The addition of $FeCl_3$ enhanced viability and growth of amebae although the number of pfu recovered was less than in heat inactivated serum.

The effect of exogenous iron and reduced pH on ameba viability was also determined in pooled human serum. In human serum not heat inactivated, no pfu were recovered after 48 h incubation, regardless of amount of iron added. The pH of human serum after addition of various iron solutions ranged from 8.1 to 8.7. In heat inactivated sera with and without exogenous iron, pfu were recovered (pH range 8.1–8.7). However, the addition of iron did not enhance viability. In a 2nd series of experiments iron was added to heat inactivated serum, and the pH was reduced to 6.2 with 1 N HCl. When this was inoculated with 48,000 pfu/ml, an enhanced recovery of pfu compared to controls was observed after 96 h (table 2).

Discussion. The results of this study show that limited growth of pathogenic *N. fowleri* can be induced in calf serum by exogenous iron. In addition, ameba viability was enhanced after 96 h incubation in heat inactivated human serum if the pH of serum was reduced to 6.2 in conjunction with iron supplements. The presence of an amebicidal heat labile factor was obvious in both sera. The addition of $FeCl_3$ moderated this factor somewhat in calf serum but was without effect in human serum.

Iron is essential for the growth of nearly all living systems. The need or affinity of *Naegleria* species for iron-containing compounds in axenic culture and in vivo has been demonstrated[3, 5, 7, 8, 12, 18]. The growth-promoting effect of iron on procaryotes in mammalian sera has been established[9, 10]. In procaryotes, this has been attributed to the role of iron as a growth-essential factor not readily acquired in serum. The addition of small amounts of iron will saturate iron chelators in serum thus making free iron available to procaryotes.

We have observed that addition of iron could maintain ameba viability and/or growth in heat inactivated human serum and in both heat inactivated and non-heat treated newborn calf serum. Growth in heat inactivated newborn calf serum was observed only when a large amount of iron (200 µg Fe/ml) was added to serum. Lesser amounts in bovine serum did not promote growth (unpublished data). Exogenous iron and reduction of pH to 6.2 greatly enhanced viability of amebae in human serum, but little or no growth occured.

In our study relatively large amounts of iron were needed to promote ameba viability and limited growth. It is questionable whether this iron acts solely as a growth factor. Addition of $FeCl_3$ (200 or 330 µg Fe/ml) did cause some temporary precipitation of serum components. The possibility that iron-induced amebic growth and viability in serum was promoted by alteration of the physical environment rather than acting exclusively as a growth factor cannot be excluded at this time. However, the iron promoted viability of amebae in serum is of considerable interest and may be of value in understanding the pathogenesis of free-living amebae.

Table 1. Number of plaque forming units (pfu) in newborn calf sera

	Serum + PAS pH 7.8	Serum + $FeCl_3$ 200 µg Fe/ml pH 6.2	Serum + HCl pH 6.2
7000 pfu/ml added			
Heat inactivated			
after 48 h	8000	29,000	0
after 96 h	6000	48,000	0
Not heat inactivated			
after 48 h	0	26,000	0
after 96 h	0	18,000	0
30,000 pfu/ml added			
Heat inactivated			
after 48 h	18,000	60,000	3000
after 96 h	6000	90,000	0
Not heat inactivated			
after 48 h	0	29,000	0
after 96 h	0	49,000	0

Values are pfu/ml.

Table 2. Number of plaque forming units (pfu) in human serum (heat inactivated) after 96 h

Serum + PAS, pH 8.7	Serum + $FeCl_3$, 330 µg Fe/ml + HCl, pH 6.2	Serum + $FeCl_3$, 20 µg Fe/ml + HCl, pH 6.2	Serum + $FeCl_3$, 2 µg Fe/ml + HCl, pH 6.2	Serum + $FeSO_4$, 330 µg Fe/ml + HCl, pH 6.2	Serum + $FeSO_4$, 20 µg Fe/ml + HCl, pH 6.2	Serum + $FeSO_4$, 2 µg Fe/ml + HCl, pH 6.2
48,000 pfu/ml added						
9400	21,000	59,000	35,000	51,000	34,000	37,000

Values are pfu/ml.

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The effect of cyproterone acetate on spermatogenesis and thumb pads of the skipper frog, *Rana cyanophlyctis* (Schn.)[1]

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Summary. The administration of cyproterone acetate (CPA) to adult male frogs of the species *R. cyanophlyctis* caused a significant reduction in the number of cell nests with primary spermatogonia (stage 0) per seminiferous tubule cross section, and a marked reduction in the height of the thumb pad epidermis and mucous glands. It is concluded that the development and/or proliferation of primary spermatogonia in *R. cyanophlyctis* are androgen dependent.

The antiandrogenic effects of cyproterone acetate (CPA) are well known in mammalian species[2], while comparative studies on lower vertebrates are limited to a few species only[3-7]. Similarly, the role of androgens in spermatogenesis is well established for mammals[8], while for amphibians the picture is at present rather confusing[9,10]. The objective of the present work was to elucidate the involvement of androgens, if any, in the spermatogenesis of the frog, *Rana cyanophlyctis*, by using CPA, which is known to bind the androgen receptor sites. In addition, the effect of CPA on Leydig cell morphology and histochemistry, and on the thumb pads (androgen dependent structure) has been investigated.

Adult male frogs *(R. cyanophlyctis)*, obtained from the surrounding areas of Dharwad in August, were used. The 1st group (8 specimens) received 0.1 ml amphibian Ringer solution and served as controls. The 2nd and 3rd groups (10 in each group) were injected with 0.1 ml amphibian Ringer solution containing 250 µg and 500 µg CPA respectively. Injections were given i.m. on alternate days for 26 days (13 injections) and animals were autopsied 24 h after the last injection. The relative weights were recorded and representative pieces of testes and thumb pads were fixed in Bouin's fluid for histological and histometric studies[11]. The remaining pieces of testes were used for the histochemical assay of the enzymes Δ^5-3β-hydroxysteroid dehydrogenase (3β-

Table 1. Effect of cyproterone acetate on the testis of *R. cyanophlyctis*

Group	Average testis weight (mg/100 g b. wt) weight ± SE	Average diameter (µm)[a] Testis	Testis tubule	Leydig cell nuclear diameter	Leydig cell Δ^5-3β-HSDH activity[b]	G-6-PDH activity[b]
Control	185 ± 25	1700 ± 18	251 ± 9	5.2 ± 0.2	+ + +	+ + + +
Treated with	189 ± 24	1630 ± 25	241 ± 6	5.0 ± 0.03	+ + +	+ + + +
250 µg CPA	NS	NS	NS	NS		
Treated with	180 ± 17	1681 ± 23	241 ± 7	5.0 ± 0.1	+ + +	+ + + +
500 µg CPA	NS	NS	NS	NS		

[a] Values ± SE; NS, nonsignificant. [b] Intensity of reaction is visually graded.

Table 2. Effect of cyproterone acetate on spermatogenesis in *R. cyanophlyctis*

Group	Number of cell nests/seminiferous tubule cross section (± SE) Stage 0	Stage I	Stage II	Stage III	Stage IV	Stage V
Control	5.38 ± 0.05	3.71 ± 0.19	2.61 ± 0.07	1.63 ± 0.12	1.3 ± 0.05	1.35 ± 0.04
Treated with	3.21 ± 0.02	3.56 ± 0.04	2.08 ± 0.16	1.80 ± 0.09	1.64 ± 0.13	1.48 ± 0.05
250 µg CPA	$p < 0.001$	NS	NS	NS	NS	NS
Treated with	3.51 ± 0.1	3.37 ± 0.11	2.49 ± 0.52	1.34 ± 0.07	1.52 ± 0.1	1.25 ± 0.06
500 µg CPA	$p < 0.001$	NS	NS	NS	NS	NS

p-Values calculated by Student's t-test between control and experimental groups. NS, nonsignificant.