where, R's are substituted groups of sulfonamides, and M's are Au, Ag, Zn, Mn or Mg.

A typical synthesis is illustrated by gold sulfadiazine: that is, 2.5 g (1 mmole) of sulfadiazine, $C_{10}H_{10}N_4O_2S$, is suspended in water at room temperature, and an equivalent sodium hydroxide, solution (0.4 g in 50 ml) is added. A solution thus obtained is stirred while 10% solution of metal salt is added. The solution turned to a brownish suspension, indicating a complex formation; and after completion of adding gold sodium chloride solution, the suspension then formed is stirred for a further 30 min at room temperature, and brownish powder is obtained after centrifugation, drying in vacuum over P_2O_5, with 80% yield. Gold and silver derivatives of sulfa drugs have been characterized by means of UV, NMR, IR and CHN analyses.

Antimicrobial property. Antimicrobial potencies of the compounds were determined as follows:

Preincubation: Medium, Trypticase soy broth (Difco).

Assay: Medium, Mueller hinton medium (Difco).

Microbial suspension of the test organism was incubated in the preincubation medium at 37 °C for 18 h, and then $^1/_{1000}$ diluted suspension obtained by the preincubation was inoculated to the test medium. MIC's of the compounds (μg/ml) for each test bacteria are listed in the table.

Among various metals attached to sulfonamides by coordination, gold and silver showed promizing antimicrobial effects. The effect is enhanced on comparing with the parent sulfonamide compounds.

Some metals are essential to living organisms, some are toxic, and others show mild biological effect. Magnesium, manganese and zinc are utilized by living organisms, so that their coordination to sulfonamides did not enhance antimicrobial property of the parent compounds. Gold and silver themselves show a mild bactericidal property, and when they are coordinated to sulfonamides, the effect is more potent than that of the parent compounds. Toxic metals such as Hg and As should be tested; but, because of their apparent toxicity, we did not use such derivatives for human application.

1 We wish to thank for the material supplied by the Central Research Laboratories, Meiji Seika Kaisha, Ltd, for antimicrobial assay.

Identification of *Trypanosoma theileri* as a contaminant in primary cultures of bovine retina

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Summary. T. theileri has been isolated from primary cultures of bovine retina and subcultered successfully for 2 passages in sub-confluent cultures. When cultures reached confluency no trypomastigotes or epimastigotes could be detected and attempts to recover trypanosomes from these cultures were unsuccessful. The presence of intracellular forms could not formally be excluded.

Although *Trypanosoma theileri* Laveran 1902, is generally nonpathogenic in the bovine, a number of fatal cases have been reported to be caused by this universally distributed organism[2]. In the host the organism appears to localize in a wide variety of organs including the central nervous system (CNS)[3]. Thus, primary cell cultures of calf spleen[4], blood lymphocytes[5], leukaemic lymph nodes[6], and fetal bovine kidney[7] have resulted in an outgrowth of both cells and trypanosomes. To our knowledge the isolation of this trypanosome from cultures of the CNS has not yet been reported. The present report describes such isolation from bovine retinal cell cultures.

Materials and methods. The external surface of bovine eyes, obtained at a local slaughter house, was sterilized by successive dipping in 70% ethanol. An incision was made at a point 1 cm caudal to the dorsal limbus and extended circumferentially parallel to the limbus to obtain 2 portions. The anterior portion was discarded. The vitreous humor was gently removed from the posterior portion to expose the underlying retina. The retina was detached with a rubber policeman from the pigmented epithelium, severed from the papilla and transferred to a centrifuge tube containing 50 ml of Eagle's minimal essential medium (MEM) (final pH 7.2).

Retinal tissue was disrupted into individual cells or groups of 2–10 cells by repeated pipetting (25 times) with a 10 ml pipette, centrifuged (400 × g for 10 min) and pelleted cells were resuspended in 100 ml of MEM + 20% fetal bovine serum (FBS), plated in 1-ml volumes in Linbro plastic tissue culture plates (FB16-24TC) and incubated at 37 °C in a humidified 5% CO_2 atmosphere. The total time elapsed between killing the animal and subsequent plating of the cells was approximately 2 h. The growth and characteristics of these retinal cells will be the subject of a separate communication[8].

When trypanosomes were detected, air dried smears of organisms, in 25% FBS, were made and stained with Giemsa. All measurements were done with an ocular micrometer; organisms were counted using an Improved Neubauer hemocytometer. Subsequent passage of trypanosomes from the primary isolate was carried out in adjacent wells or in freshly prepared cultures. Inocula consisted of 50 μl aliquots of a suspension containing cells and trypanosomes that had been obtained by trypsinization of infected monolayers.

Results and discussion. Routine sterility control on the 2nd day of culture revealed no microbial contaminants. 6 days post plating marked acidity was noticed in 1 of the culture wells; examination of the culture fluid revealed large numbers of motile, flagellated organisms. Inspection of wet mounts under phase microscopy (magnification: × 800) led to a tentative identification of the organism as *T. theileri.* To expedite verification, trypanosomes in stained smears were subjected to micrometer measurements. These results are summarized in the table and the data are comparable to that of others[2,9]. On the basis of these morphological criteria we conclude that the organism is *T. theileri.*

Examination of the forms of the organism in the prelimi-

nary isolate showed that both the crithidial (epimastigote) and the trypanosome (trypomastigote) forms were present (figure 1). It had been previously noted that an increase in crithidial forms in vitro was associated with a marked drop in the pH (5.5–6.0) of the culture[10]. A similar phenomenon was also observed in this study as multiple trypanosomal forms were favoured in the presence of high levels of lactate in the media as evidenced by pH changes. Since erythrocyte energy metabolism is characterized by anaerobic glycolysis and the concomitant build-up of high intracellular concentrations of lactate and other glycolytic intermediates[11] it may explain why previous media used for the cultivation of *T. theileri* had required supplements of erythrocytes or their dried lysates[10]. A comparable capacity of retinal tissue to carry out both aerobic and anaerobic glycolysis[12] may be of some relevance to these observations. In this respect retinal tissue metabolism resembles that of embryonic tissue, tumor cells and leukocytes[13] in that a

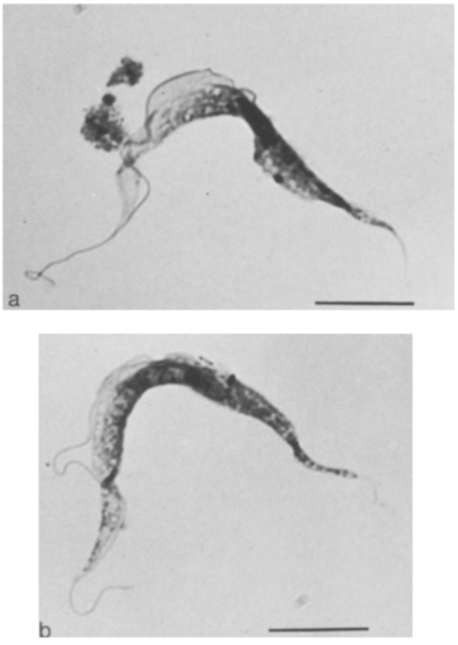

Fig. 1. *a* Trypomastigote form of *T. theileri* grown in the bovine retinal cell culture system. *b* Epimastigote, or transitional form of *T. theileri* from the same culture. Stained Giemsa; scale bar 10 µm.

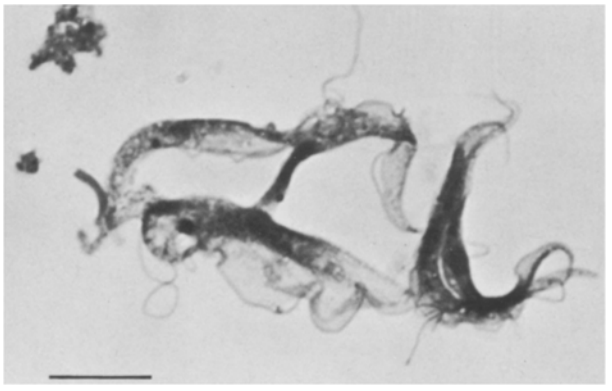

Fig. 2. Dividing forms of *T. theileri* grown in the bovine retinal cell culture system at an FBS concentration of 25%. Stained Giemsa; scale bar 10 µm.

Morphometric characterization of *T. theileri* cultivated in vitro*

Parameter	Distance (µm)**
Posterior end to kinetoplast (PK)	15.26 ± 0.56 (5.17–38.73)***
Kinetoplast to centre of nucleus (KN)	6.80 ± 0.34 (1.72–17.24)
Centre of nucleus to anterior end (AN)	22.76 ± 0.57 (8.62–36.20)
Body length without flagellum (L)	44.83 ± 0.80 (27.58–64.65)
Length of flagellum	20.75 ± 0.94 (5.17–46.55)
Ratio: $\frac{PK}{L}$	0.34 (0.24–0.40)
Ratio: $\frac{AN}{L}$	0.50 (0.44–0.56)

*Measurements were carried out on 100 organisms. **Mean ± SEM. ***Range of respective parameter in parentheses.

Crabtree effect may be demonstrated[14]. Therefore, it is not merely coincidental that *T. theileri* has already been found to be associated with these latter cell types[4–7]. This report then adds to what appears to be a consistent pattern that described the parasite's preference for an environment containing a particular type of host cell, viz., one characterized by specific metabolic features.

In the course of serial subculture (inocula contained < 10 organism/ml) some of the nutritional requirements of *T. theileri* were determined by observing growth over a 1-week period in the presence of different FBS concentrations (5–40% FBS in 5% increments). Dividing forms (figure 2) were quite evident and maximum number of trypanosomes (1.5×10^4/ml) were obtained in the range 20–25%; similar requirements on the FBS requirements of *T. brucei* in a bovine fibroblast-like cell system have recently been made[15]. Decreasing the serum concentration to 5% or lower resulted in a reduced number of trypanosomes ($< 1.5 \times 10^3$/ml) possibly as a result of nutritional limitations. Increasing the concentration to 40% also decreased the number of organisms ($< 5.0 \times 10^3$/ml) as a result of the cells reaching confluency very rapidly and the concomitant disappearance or development of intracellular forms under these conditions. This occurred even at 20% FBS when cultures approached confluency.

Whether this disappearance signified the establishment of an occult phase[16] could not be determined as attempts to recover trypanosomes from confluent cultures were invariably unsuccessful even though inocula from infected confluent cultures were passaged on subconfluent monolayers. In one instance a few trypomastigotes appeared but failed to multiply. However, confluent cultures suspected of being latently infected invariably began to show signs of degeneration after prolonged cultivation (4–7 weeks). In contrast, confluent control cultures from uninfected retina did not exhibit comparable changes. These infected monolayers are currently being examined by electron microscopy to establish if intracellular forms are present.

The isolation of *T. theileri* from bovine retina is in itself significant. The retina develops during the embryonic period as an ectopic portion of the primitive forebrain, and in the adult retains its resemblance to the CNS[17]. Glucose is the prime substrate of retinal metabolism with observed rates of glucose and oxygen utilization exceeding that of cerebral cortex[18]. It appears then, the cultivation of this organism in a retinal cell culture system may be adequately representative of metabolic conditions found within the CNS. Furthermore, it would appear that the levels and type of carbon source may determine the development of *T. theileri* both in vivo and in vitro. Investigations are continuing in our laboratories to clarify the relationship between nutritional factors in media and the subsequent development of *T. theileri*.

1 We wish to thank Dr S. Dershko, Division of Meat Inspection, Health of Animals Branch, Department of Agriculture (Canada) and Intercontinental Packers, Ltd. for their assistance in obtaining the freshest possible specimens. The skillful technical assistance of Mrs J. Graham is also gratefully acknowledged. This investigation was supported by the M.R.C. of Canada.
2 P. Wood, M.A. Soltys and A.C. Gillick, Can. J. comp. Med. *34*, 142 (1970).
3 J. Carmichael, Parasitology *31*, 498 (1939).
4 J.E. Moulton and H.H. Krauss, Cornell Vet. *602*, 124 (1972).
5 W.A. Malmquist, Vet. Rec. *77*, 350 (1965).
6 W.C.D. Hare and E.J. Soulsby, J. Parasit. *55*, 973 (1969).
7 B.D. Lundholm, J. Storz and D.G. McKercher, Virology *8*, 394 (1959).
8 M.W. Warenycia and L.A. Babiuk, in preparation.
9 C.A. Hoare, The Trypanosomes of Mammals. Blackwell Scientific Publications, Oxford 1971.
10 E.J. Splitter and E.J.L. Soulsby, Expt. Parasit. *21*, 137 (1967).
11 G.J. Brewer, in: The Red Blood Cell, 2nd ed., vol. 1, p. 391. Ed. D. MacN. Surgenor. Academic Press, New York 1974.
12 O. Warburg, Biochem. Z. *184*, 484 (1927).
13 L.H. Cohen and W.K. Noell, J. Neurochem. *5*, 253 (1960).
14 L.H. Cohen and W.K. Noell, in: Biochemistry of the Retina, p. 36. Ed. C.N. Graymore. Academic Press, New York-London 1965.
15 H. Hirumi, J.J. Doyle and K. Hirumi, Science *196*, 992 (1977).
16 C. Brack, Acta trop. *25*, 289 (1968).
17 D.G. Cogan, Neurology of the Visual System. Charles C. Thomas, Springfield, Ill., 1966.
18 F.H. Adler, in: Physiology of the Eye, 3rd ed., p. 558. C.V. Mosby Co., St. Louis 1965.

A rapid method for the fractionation of avian blood cell nuclei[1]

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Summary. Red blood cell nuclei from avian anaemic blood have been fractionated by rate sedimentation on discontinuous sucrose gradients into fractions which can be distinguished both by their RNA synthesizing ability and by their morphology as revealed by electron microscopy.

Anaemic avian blood contains both mature erythrocytes and a variable fraction of predominantly late polychromatic erythrocytes[2,3]. Avian red blood cell fractions, corresponding to different stages of development have been isolated by several workers[2-5] making use either of sedimentation in a density gradient or by separating layers from a packed cell mass after centrifugation. For subsequent preparation of nuclei a lengthy cell fractionation procedure may give rise to artefacts. A different approach is described here viz. nuclear isolation and subsequent fractionation which leads to a rapid method to prepare nuclei of distinct activity and morphology.

Materials and methods. Anaemic blood was obtained from White Leghorn cocks after phenylhydrazine injection as described before[6]. All preparations were carried out at 0-4 °C unless otherwise stated. Blood cells were washed 3 times by centrifugation at 10,000 × g for 2 min in 10 vol. 0.12 M NaCl containing 5 mM KCl and 5 mM $MgCl_2$. The buffy coat was removed taking care to remove as few red cells as possible. Cells were lysed at 0 °C for 30 min in 10 vol. 0.05% (w/v) Saponin (Merck, white pure) made up in a 0.25 M sucrose, 0.12 M NaCl, 1.5 mM $MgCl_2$, 15 mM Tris-HCl, pH 7.4 buffer (buffer A). A nuclear pellet was obtained from this suspension by centrifugation at 3000 × g for 5 min. The nuclei were washed with buffer A by centrifugation as above. 2 types of discontinuous sucrose gradients were prepared as follows:

Gradient A: Equal quantities of sucrose solutions (2.0/1.9/1.85/1.8/1.75/1.7 M sucrose in buffer A) were layered into the 34-ml-capacity tube of the Beckman SW 25.1 rotor. Nuclei ($2-5 \times 10^8$) were layered carefully onto this gradient and spun at 2500 × g for 30 min. 4 fractions, each comprising nuclei at an interface and the sucrose layer above it, were collected with a total recovery of nuclei of 91%. No nuclei were recovered from the 1.70/1.75 M interface.

Gradient B: Equal quantities of 2.0/1.75/1.5 M sucrose solutions were layered into the 250-ml-capacity tubes of the Sorvall HS-4 rotor, $2-5 \times 10^9$ nuclei were layered onto the gradient and spun at 2500 × g for 15 min. 2 fractions comprising 68% of the total nuclei, were collected from the 2 sucrose interfaces. The remaining nuclei could be recovered from the 1.75 M sucrose layer (19%), sample layer (6%) and walls of the tube (7%). The above fractions were collected by syringe or Pasteur pipette, suspended in 5 vol. buffer A, spun at 8000 × g for 15 min and the pellet finally resuspended in buffer A. Nuclei were counted in the improved Neubauer haemocytometer. RNA synthesizing activity of nuclei was determined essentially as described before[6] (incubation with 5 μCi [^{3}H]-UTP using $40-60 \times 10^6$ nuclei and 0.05 M ammonium sulphate and 0.05 M Tris-HCl pH 7.4). The reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid and the sample prepared for counting as described before[6].

Results and discussion. An initial study of the size of nuclei in stained whole cell preparations of anaemic blood showed appreciable differences between mature erythrocytes and cells with basophilic granules typical of reticulocytes. Using the formula given by Austoker et al.[7] the mean nuclear volume of stained mature erythrocyte nuclei and

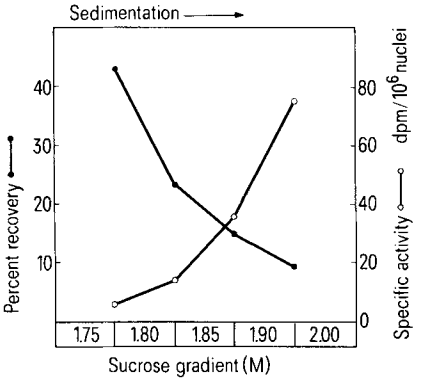

Fig. 1. Fractionation of avian anaemic red blood cell nuclei on a discontinuous sucrose gradient. RNA synthesizing activity and recovery in 4 fractions separated on gradient A are shown. The major part of each fraction was recovered from the interfaces as indicated. Recovery is expressed as percentage of nuclei applied to gradient. Specific activity is expressed as incorporation of [^{3}H]-UTP into RNA per 10^6 nuclei. Incubation conditions were as given in text except that ammonium sulphate concentration was 0.4 M.