marine resources as intensively as their LSA successors has already come from analyses of vertebrate remains from MSA and LSA coastal caves in the southern Cape (*11*). Thus, the MSA deposits at Klasies River Mouth and in the probably early Last Glacial levels of Die Kelders Cave (*12*) contain numerous seal and penguin bones but very few remains of fish and flying birds. In LSA sites with comparable numbers of seal and penguin bones, remains of flying birds and of fish are abundant to superabundant (*13*). The most economical explanation for this contrast is that MSA peoples were technologically incapable of active fishing and fowling. Implements such as gorges and line (or net) sinkers, which are reasonably interpreted as fishing and fowling gear, are so far known only in LSA contexts.

In sum, then, the Middle Stone Age open-air shell middens at Sea Harvest and Hoedjies Punt not only contain some of the earliest evidence in the world for marine resource utilization but, in combination with evidence from other sites, they suggest that such utilization was less intensive during the Last Interglacial and earliest Last Glacial than in the terminal Pleistocene and Present Interglacial.

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14. I thank D. M. Avery, G. Avery, R. G. Klein, and J. E. Parkington for faunal identifications and A. W. Fairhall for radiocarbon determinations. I thank R. G. Klein, K. W. Butzer, and L. G. Freeman, who made helpful comments on an earlier draft of the manuscript. This work was supported by grant BNS 76-82734 from the National Science Foundation and the Hinds Fund, Committee on Evolutionary Biology, University of Chicago.

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Formation of Mutagens in Beef and Beef Extract During Cooking

Abstract. *Mutagens, distinguishable from benzo*[a]*pyrene and from mutagenic amino acid and protein pyrolysis products, are formed when ground beef is cooked in a home hamburger cooking appliance or when beef stock is concentrated, by boiling, to a paste known commercially as beef extract. "Well-done" hamburgers contain about 0.14 part per million of the mutagens, and beef bouillon cubes which contain beef extract about 0.1 part per million. Since such mutagens may be potentially carcinogenic and are formed during ordinary cooking procedures, their occurrence raises questions about possible risks to human health.*

It has become evident that environmental agents are responsible for much of the incidence of cancer in populations in the United States and elsewhere (*1*). Efforts to study such agents have been enhanced by the development of Ames' method for detecting mutagens in a system based on histidine-negative strains of *Salmonella typhimurium* (*2*). The method is rapid and inexpensive, and because there is a significant empirical relation between mutagenic activity in the Ames system and carcinogenicity (as determined by tumor incidence in laboratory animals) (*3*), it can be used for screening environmental agents that may be carcinogenic in human beings.

During an investigation of a minor anomaly in the Ames test, we discovered that certain commercial media that are widely used for culturing bacteria, including Ames' *Salmonella* strains, contain active mutagens (*4*). The anomaly is a small but consistent increase in the background rate of mutation in strains TA 1538 and TA 98, both of which are particularly sensitive to frame-shift mutations, when they are grown in the presence of the S-9 microsome preparation. This preparation is obtained by centrifugation of a liver homogenate at 9000*g*, and is used in the Ames system to convert certain test substances to mutagens. We have shown that the medium in which the bacterial inoculum is grown contains substances that are mutagenic in the presence of the microsome preparation. The mutagens occur only in media such as Bacto nutrient broth (Difco) that contain beef extract, a product obtained when beef stock (prepared by boiling beef tissue in water, filtering off the tissue, and removing the fat) is boiled down to 20 percent or less of its original volume. Beef extract is commercially available and is used in certain food preparations such as bouillon cubes (*5*).

We report here studies designed to determine the origin of the mutagens detected in bacterial media that contain beef extract. Beef stock was prepared by boiling 500 g (wet weight) of lean ground beef in two volumes of distilled water. Solids were removed by filtration, the liquid was cooled, and solidified fat was skimmed off. The beef stock was then boiled in an open beaker for 10 hours. At intervals, portions [each representing 0.69 g of the original beef stock (dry weight)] were analyzed by the method used earlier to detect mutagens in bacterial nutrients (based on methylene chloride extraction; see legend to Fig. 1). An initial sample yielded an average of 21 revertant colonies per plate, not significantly different from the control value of 29 revertants per plate. After boiling for 8.5 hours, the beef stock had been reduced to 40 percent of its original volume, and a portion yielded an average of 181 revertants per plate. After 10 hours of boiling, the beef stock was reduced to a dark brown paste (similar in appearance to commercial beef extract) representing about 5 percent of the original volume, and a portion yielded an average of 1572 revertants per plate. Thus, the mutagens are not present in either beef tissue or beef stock, but are produced when the latter is boiled extensively and reduced in volume to form beef extract, thus raising activity per unit of dry weight (*6*).

A dose-response curve obtained from methylene chloride extracts representing increasing portions of a commercial beef extract preparation [Bacto beef extract (Difco)] is shown in Fig. 1. It is evident that this material contains mutagens that are active only in the presence of the microsome preparation. Similar dose-response curves show that, in the presence of the microsome preparation, the mutagens extractable from this source by methylene chloride are equally active to-

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ward strain TA 98, about one-fourth as active toward strain TA 1537, and inactive toward strains TA 100 and TA 1535.

Since the mutagens present in beef extract are produced in conditions that may be encountered in cooking procedures it was of interest to determine their occurrence in cooked beef. Lean ground beef (in 100-g portions, wet weight) was cooked in an electrically heated (plate temperature 200°C) home hamburger cooking appliance for 1.5 minutes, 3.0 minutes, or 5.5 minutes, that is, rare, medium, or well-done. The cooked samples and an uncooked control were homogenized in twice their volume of distilled water in a Waring blender, and the homogenates treated as described in the legend for Fig. 2. Portions of the final methylene chloride extracts representing 5 and 25 g (dry weight) of the cooked beef (in the case of the uncooked control, portions representing 5, 10, and 35 g were tested) were dried, taken up in dimethyl sulfoxide (DMSO), and tested in the usual way against strain TA 1538 in the presence and absence of the microsome preparation. The results are shown in Fig. 2. The mutagenic activities of all samples tested in the absence of the microsome preparation fell within the range of control values. The values obtained from the uncooked sample in the presence of the microsome preparation were slightly above the control value, but the increase is of doubtful significance. However, all the cooked samples yielded substantial mutagenic activity. The values increased with cooking time, the "well-done" samples yielding the highest values: 954 revertants per plate with a 5-g sample and 3388 revertants per plate with a 25-g sample.

These data suggested a possible relation between our observations and earlier evidence that mutagens, including known carcinogens such as benzo[*a*]pyrene, are formed in meat and fish during certain cooking procedures. Thus, Sugimura *et al.* (*7*) reported that condensed smoke from meat and fish broiled over an open gas or charcoal flame contains material that is mutagenic toward strain TA 98, usually only in the presence of the microsome preparation. They reported that the mutagenic activity was much too high to be accounted for by the amounts of benzo[*a*]pyrene present in the smoke condensates and suggested that other mutagens may arise from pyrolysis of tissue protein and amino acids. This suggestion was based on their observation that pyrolysis (at temperatures of 300° to 600°C) of proteins and certain amino acids produces mutagens similar in their effects in the Ames' test to those observed in the smoke condensates (*8*). They also reported similar activity in material obtained from the charred surface of a broiled beef steak.

In view of the foregoing results, it was of interest to compare the mutagens that occur in beef extract and cooked beef with those formed by pyrolysis of amino acids and protein, and with benzo[*a*]pyrene. Methylene chloride extracts of beef extract, cooked beef, cooked beef with added benzo[*a*]pyrene, a pyrolyzed mixture of amino acids, and pyrolyzed histone were chromatographed on a glass fiber sheet impregnated with silica gel (Gelman ITLC-SG). Successive 1-cm zones of the developed chromatograms were extracted in chloroform : methanol (90 : 10 by volume) and dried. The samples were then taken up in DMSO and tested on strain TA 1538 in the usual way. Since the dose-response curve for the beef extract mutagens is linear (*9*) (see Fig. 1, but note that the plot is semilogarithmic) over mutation rates ranging up to several thousand revertants per plate, rates observed within that range in the successive zones of a chromatogram reflect the relative amounts of mutagens present. Figure 3 shows results of such analyses of methylene chloride extracts of Bacto beef extract and of a beef patty cooked for 10 minutes on a ceramic hot plate (*10*); a mixture of benzene and methanol (95 : 5 by volume) was used as the chromatographic solvent. The mutagens present in the two samples exhibit identical chromatographic behavior, with a major peak at an R_F 0.5 and a slight shoulder at R_F 0.3. Figure 4A shows the results of a similar analysis (in which 100 percent hexane was used as the chromatographic solvent) of methylene chloride extracts of Bacto beef extract, a beef patty cooked on a hot plate, and a patty to which benzo[*a*]pyrene (25 μg/kg, wet weight) had been added (after

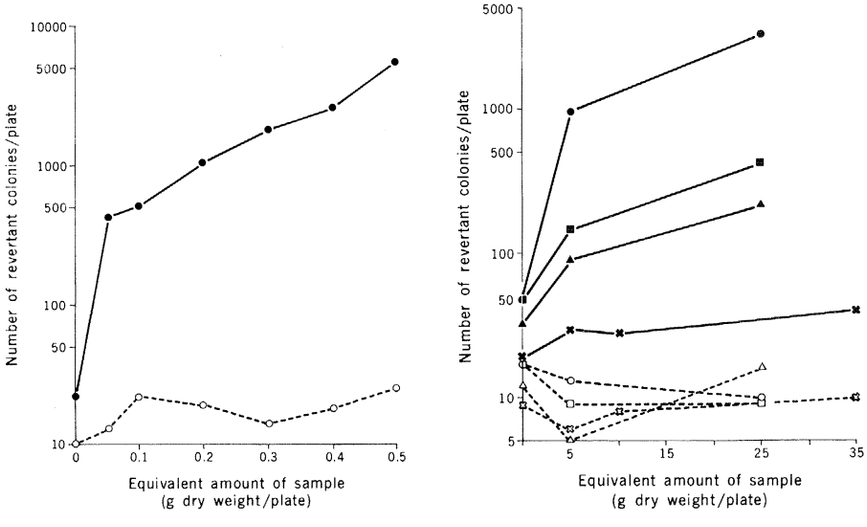

Fig. 1 (left). Dose-response curves of methylene chloride extracts of Bacto beef extract (Difco) tested on histidine-negative strain TA 1538 of *Salmonella typhimurium*, inoculated onto a synthetic agar medium, together with trace amounts of histidine and biotin (2 ml of a 0.05 m*M* solution), in accordance with the procedure of Ames *et al.* (*2*). Ordinate: number of histidine-positive revertants per plate. Abscissa: amount of sample used to prepare the methylene chloride extract added per plate. Solid lines and filled symbols represent plates to which 0.5 ml of a standard S-9 microsome preparation (obtained from the livers of rats injected with polychlorinated biphenyl) was added. Dotted lines and open symbols represent plates to which the microsome preparation was not added. To prepare samples, Bacto beef extract was dissolved in water and acidified (to *p*H 2.0) with HCl. Protein was then precipitated by adding ammonium sulfate to saturation; the sample was then filtered through glass wool, and extracted twice with methylene chloride. The aqueous phase was adjusted to *p*H 10 with ammonium hydroxide and extracted three times with methylene chloride and the pooled basic extracts evaporated to dryness. The residue was taken up in 0.25 ml of DMSO and added to the plates. After 48 hours of incubation at 37°C, the number of reverant colonies on duplicate plates was counted. All results are reported as the averages of the duplicate plates. Fig. 2 (right). Dose-response curves for methylene chloride extracts of uncooked and cooked lean ground beef. Tests were conducted as described in the legend for Fig. 1 in the presence (filled symbols) or absence (open symbols) of the microsome preparation. Samples (100 g, wet weight) of lean ground beef were tested before (crosses) and after cooking in an electrically heated home hamburger cooking appliance for 1.5 minutes (triangles) 3.0 minutes (squares), or 5.5 minutes (circles), that is, rare, medium, or well-done, respectively. After cooking, the beef patties were homogenized in two volumes of water in a Waring blender. The acidification, filtration, extraction, and plating procedures were the same as those described in Fig. 1.

cooking and extraction). All of the mutagenic activity associated with Bacto beef extract, and cooked beef remained at the origin, while the sample of the latter to which benzo[*a*]pyrene had been added exhibited an additional peak at R_F 0.85. Figure 4B shows a similar chromatographic analysis (with hexane and acetone, 50 : 50 by volume, as the chromatographic solvent) of a methylene chloride extract of Difco beef extract. This is compared with methylene chloride extracts of the pyrolysis product (pyrolysis temperature 350°C) of a mixture of 2 mg of each of the 18 amino acids which yield mutagenic material when pyrolyzed, and of pyrolyzed histone, which yields similar mutagenic material (*8, 11*). The material from Bacto beef extract exhibited a peak at R_F 0.4. In contrast, the mutagenic material from the pyrolyzed amino acids exhibited a main peak at R_F 0.8, a minor peak at R_F 0.6, and some residual activity at the origin. The material from the pyrolyzed histone migrated to the solvent front, with a shoulder between R_F 0.5 and 0.9 and some activity at the origin. There was no activity (above the control value) in the region in which the peak, due to the beef extract mutagens, occurred.

These analyses indicate (i) that the mutagens produced when beef stock is heated to form beef extract are chromatographically indistinguishable from those produced when ground beef is cooked on an electrically heated hot plate, and (ii) that the mutagens are chromatographically distinguishable from both benzo[*a*]pyrene and the mutagens produced from pyrolyzed amino acids and protein. Further studies of the mutagenic material extractable by methylene chloride from Bacto beef extract, partially purified by successive thin-layer chromatographic separations, show that: (i) the mutagen is a basic substance, extractable by organic solvents from aqueous solutions at alkaline *p*H; (ii) it is unaffected in its mutagenic activity or chromatographic behavior by refluxing in 6*N* HCl for 6 hours; and (iii) on treatment with nitrous acid the material becomes inherently mutagenic (that is, in the absence of the microsome preparation), suggesting the possible formation of a nitroso group. The conditions under which these mutagens are formed are similar to those characteristic of the Maillard or "browning" reactions, in which amino acids and sugars react to produce a variety of complex substances (*12*).

We have also found these mutagens in two commercial food preparations, which, according to their labels, contain beef extract. A 5-g (dry weight) sample of beef bouillon cubes analyzed as described above (on strain TA 1538) yielded 151 revertants per plate, and a 5-g (dry weight) sample of a "gravy concentrate" yielded 407 revertants per plate.

The foregoing experiments show that one or more substances which are mutagenic in the Ames system (in the presence of the microsomal preparation) are produced when beef stock is heated and condensed to form beef extract and when ground beef is cooked (at temperatures not exceeding 200°C) on an electric hot plate or a home hamburger cooking appliance. These mutagens are neither benzo[*a*]pyrene nor the mutagenic substances produced when amino acids or protein are pyrolyzed. This is indicated by the chromatographic analyses reported herein. Moreover, according to Matsumoto *et al.* (*8*) the mutagenic py-

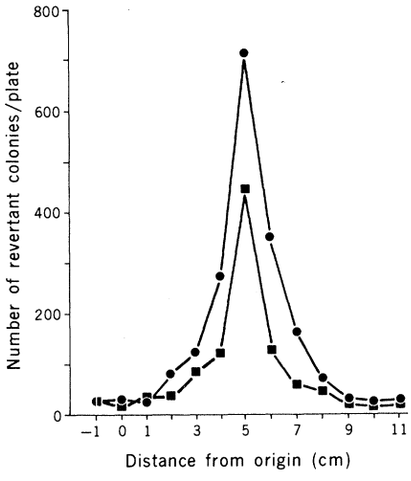

Fig. 3. Thin-layer chromatographic fractionation of the methylene chloride extracts of Bacto beef extract (circles) and hot plate cooked lean ground beef (squares). Gelman ITLC-SG sheets were used with benzene and methanol (95 : 5 by volume) as the solvent system. Four ground beef patties (each approximately 120 g, wet weight) were wrapped in aluminum foil and cooked on a 350°C ceramic hot plate for 10 to 12 minutes. Thermocouples at the surface of a patty and in its interior recorded temperatures of 200°C and 80°C, respectively, at the end of the cooking period. Extracts of the cooked patties were prepared as described in the legend for Fig. 2, those of Bacto beef extract as described in the legend for Fig. 1. Extracts equivalent to approximately 0.2 g of beef extract and 26 g (dry weight) of ground beef were applied to the chromatogram. One-centimeter zones of the developed chromatogram were extracted with chloroform and methanol (90 : 10 by volume). Portions were taken to dryness, resuspended in DMSO, and tested on strain TA 1538 in the presence of the microsome preparation according to procedures described in the legend for Fig. 1.

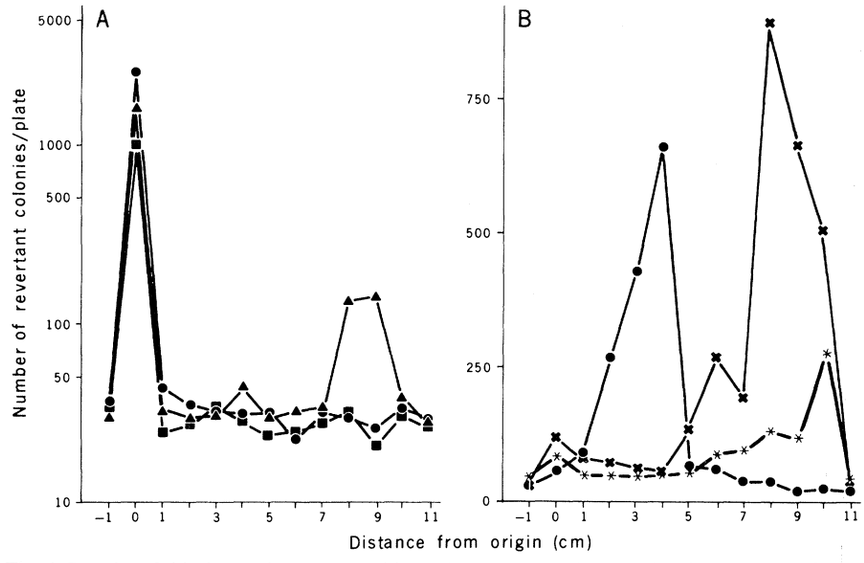

Fig. 4. Results of thin-layer chromatographic analyses. (A) Methylene chloride extracts of Bacto beef extract (filled circles), beef cooked on a hot plate (squares), and beef with added benzo[*a*]pyrene (25 μg/kg, wet weight) cooked on a hot plate (triangles). Extracts equivalent to approximately 0.2 g of beef extract and 26 g of ground beef (with and without benzo[*a*]-pyrene) were applied to the chromatogram. The chromatographic solvent was 100 percent hexane. (B) Methylene chloride extracts of Bacto beef extract (circles), a mixture of 18 amino acids pyrolyzed at 350°C (crosses), and calf thymus histone (Sigma) pyrolyzed at 600°C (asterisks). Extracts equivalent to approximately 0.2 g of beef extract, 9 mg of amino acids (equal weights of each), and 60 mg of histone were applied to the chromatogram. In each case, 1-cm zones of the chromatograms were extracted with 10 ml of chloroform, and 1-ml portions were dried, taken up in DMSO, and added to test plates. The chromatographic solvent was hexane and acetone (50:50 by volume). Chromatogram zone extraction and mutagenic analysis were as described in the legend for Fig. 3. The microsome preparation was present.

rolysis products are formed only at temperatures in excess of 300°C, which can readily occur in foods cooked over open flames. In contrast, the mutagens that we have detected in beef extract are produced at temperatures which do not exceed 105°C, whereas those detected in cooked ground beef are produced at temperatures that do not exceed 200°C. Thus, these mutagens are produced in conditions which occur in common cooking procedures, including the preparation of hamburgers on electrically heated hot plates at conventional cooking temperatures and times.

The mutagens found in beef extract and cooked beef are relatively active compared with a typical mutagen, 2-acetylaminofluorene (AAF), which is also active toward strains TA 1538 and TA 98. Tested on strain TA 1538, 50 μg of AAF (which is in the linear portion of the dose-response curve) yields about 4800 revertants per plate. Active material prepared from a bacterial medium containing 37 percent beef extract yielded 1367 revertants per plate containing 3.85 μg (in the linear part of the dose-response curve) of a preparation partially purified, by successive chromatographic fractionation, from the original methylene chloride extract. Accordingly, the specific activity of the beef extract mutagen is a minimum of about 350 revertants per plate per microgram, compared to 96 revertants per plate per microgram for AAF. On the basis of the estimated 350 revertants per plate per microgram for impure beef extract, a 3.6-g beef bouillon cube contains a minimum of approximately 0.3 μg of mutagen and a 100-g (wet weight) lean-beef hamburger contains approximately 1 to 14 μg of mutagen, depending on the extent of cooking. These figures correspond to concentrations, on a wet weight basis, of 0.1 part per million of mutagen in beef bouillon cubes and from 0.01 to 0.14 part per million in cooked hamburgers.

If, as indicated by the observed correlation between mutagenicity in the Ames test and carcinogenicity, these mutagens—once purified and tested on laboratory animals—are found to be carcinogens, their apparent concentration in some foods may represent an appreciable risk to certain populations. The relatively ordinary circumstances in which these mutagens are formed suggest that they may arise during the course of certain conventional cooking procedures, in addition to the preparation of hamburgers, such as the braising of beef and the evaporation of beef stock in the preparation of stews. However, the sensitivity of the effect of cooking times (see Fig. 2) suggests that it may be possible to modify cooking procedures in ways that reduce the formation of the mutagens.

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5. To avoid confusion, we retain in this report the commercial definition of beef extract, although in a more technical sense it is actually a condensed preparation of beef stock, which is itself an aqueous extract of beef.
6. Whenever a sample derived from biological material appears to induce mutagenic activity in the Ames system it is important to consider the possibility that the effect may be due to the presence of histidine in the sample. (Histidine would enable nonmutant histidine-negative cells to form colonies, giving the appearance of enhanced reverse mutation.) That the effects we describe are not due to the presence of histidine in the samples is shown by the following: (i) Replica plating of test plates show that the colonies are comprised of revertant, histidine-positive cells; (ii) the effect consistently requires the presence of the microsome preparation, a condition not required for the influence of histidine on colony formation; (iii) control experiments show that the extraction and chromatographic procedures which we employ eliminate histidine from the material finally added to the test plates.
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10. From thermocouples at the surface of a patty and in its interior, it was determined that the maximum temperature (at the end of the cooking period) at the surface of the patty was 200°C and in the interior, 80°C.
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Unusual Antibody-Induced Modulation of Surface Antigens in the Cell Coat of a Bloodstream Trypanosome

Abstract. *Unlike other eukaryotic cells,* Trypanosoma lewisi *forms caps at 0°C when incubated with rabbit immunoglobulin G (IgG) directed against surface IgG from the rat host. The host IgG, which is specific for parasite antigens, probably does not cause capping of these antigens in vivo, since trypanosomes treated with Fab fragments directed against rat IgG are uniformly labeled and do not cap at 0°C or 37°C.*

The formation of dense aggregates or caps of host immunoglobulin G (IgG), a surface component of the rat parasite *Trypanosoma lewisi*, is not inhibited at 0°C. The diffuse or patchy surface staining typical of labeling with bivalent antibody at low temperatures in the absence of metabolic inhibitors rarely occurs; more than 90 percent of the cells observed are capped immediately after labeling with fluorescein-conjugated rabbit antibody to rat IgG. The caps are usually located along the undulating membrane, or at the posterior of the trypanosomes, or both; bipolar caps are rarely seen. The caps do not reflect the distribution of host antibody in vivo, since trypanosomes are uniformly labeled by Fab fragments directed against rat IgG.

In contrast to *T. lewisi*, the antibody–surface antigen complexes of other eukaryotic cells are observed to first form small aggregates (spots or patches), and later, at temperatures above 0°C, larger, usually polar, dense aggregates (caps) form. After incubation with appropriately labeled antibodies, lymphocyte surface immunoglobulins (*1*) and HLA antigens (*2*) form caps by an energy-dependent redistribution process that is inhibited both by temperatures near 0°C and by metabolic inhibitors. Inhibition of capping at 0°C has also been shown for the protozoan parasites *Leishmania enriettii, L. tropica* (*3*), *L. donovani* (*4*), and *T. brucei* (*5*); in these organisms, the initial fluorescence is uniform and diffuse.

Trypanosoma lewisi has a surface coat composed partially of bound host serum

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