In contrast, the 4 conifers that have high maltol contents have a narrow vertical distribution. They are found only in the subalpine zone as already stated. They are the oldest plants that were already distributed and flourished prior to the great ice age in Honshu. Our knowledge of geographic history teaches us that there was a familiar exchange between the conifers and circumpolar plants of the old continent before Honshu was separated from the continent[2].

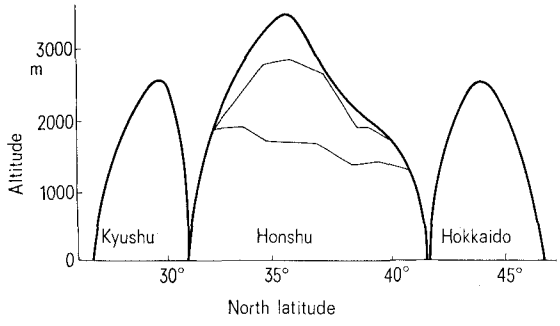

Fig. 1. The vertical distribution of *Abies mariesii, A. veitchii, A. homolepsis* and *Tsuga diversifolia* (Y. HAYASHI).

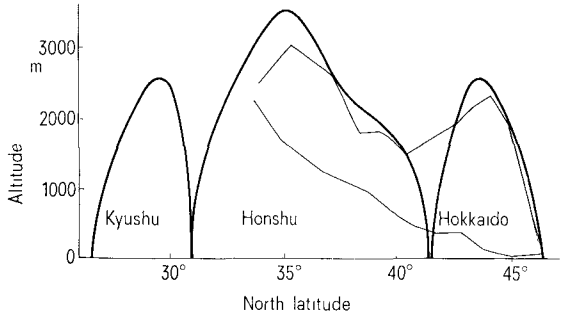

Fig. 2. The vertical distribution of *Pinus pumila* (Y. HAYASHI).

The leaves of the 4 species of conifers that have high maltol contents are also marked by high sugar contents, which protect them against freezing[3]. In addition, the lower epidermises of these leaves are coated with a white waxy layer in which maltol is abundant. Maltol has sweet odour that is often used as a flavour enhancer and, as a result, these 4 species have a characteristic sweet smell.

Because maltol can be produced simply[4] by heating sugar, by fermenting malt, or by cooking food, it is believed that maltol may be biosynthesized from sugars in plants. At any rate, there seems to be a definite connection between the presence of sugars and maltol in the leaves of the 4 species of conifer. In particular, it is interesting from the viewpoint of plant ecology that high amounts of maltol and sugars are contained in leaves of these conifers that ranged only in a subalpine zone in Japan.

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The Generation of Toxic Activity from *Trypanosoma congolense*[1]

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Summary. Trypanosoma congolense organisms, on incubation at 20 °C for $9^1/_2$ h, were found to generate phospholipase-like activity which was capable of mediating lysis of both nucleated cells and erythrocytes as well as an acute inflammatory response on intradermal inoculation.

Although it was suggested long ago that salivarian trypanosomes may produce 'toxins'[2,3], evidence for the existence of these has generally been fragmentary and inconclusive. The toxic activities which have been demonstrated in trypanosome preparations from various species include, the capacity to cause acute inflammation on intradermal inoculation[4] and around intraperitoneal diffusion chambers[5], an ability to induce mild narcosis in mice[6], and the production of hemolytic factors[7,8]. Nevertheless, none of these have been unequivocally established as playing a significant role in disease due to the African trypanosomes.

In this communication we report on the generation of toxic activity from *T. congolense* (Strain TREU 112) which we consider to be of potential significance in the pathogenesis of disease caused by this organism. During the course of experiments on dermal hypersensitivity to *T. congolense* in rabbits it was observed that a single intradermal injection of 0.05 ml of a 1.0% v/v suspension

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of washed trypanosomes in phosphate buffered glucose[9] pH 8.0 which had been maintained at 20 °C for 24 h (during which time the pH dropped to 5.5) gave rise to a local acute inflammatory reaction in normal, unsensitized animals. A similar suspension of freshly isolated organisms did not do this. This skin reaction reached maximum intensity 5 h after injection. Histologically, the injection site showed vascular dilatation, edema and perivascular neutrophil infiltration.

When the skin reactive material was tested in vitro it was found to be potently hemolytic and cytotoxic. Thus 1×10^9 trypanosomes (freshly isolated from rat blood and purified by passage through DEAE cellulose[9]) were suspended in 0.5 ml phosphate buffered glucose, mixed with an equal volume of 2.5% washed sheep erythrocytes also in phosphate buffered glucose and incubated at 20 °C. Rapid and complete hemolysis (as measured spectrophotometrically by absorption at 412 nm indicating the release of free hemoglobin into the supernatant fluid) occurred after approximately nine hours (Figure 1) by which time all organisms were dead and autolysed.

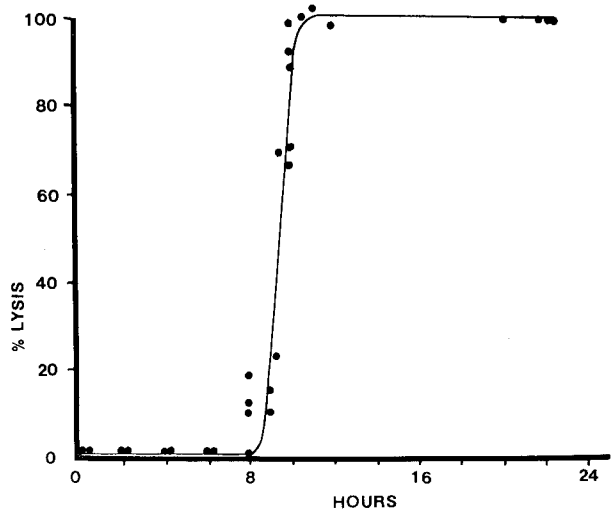

Fig. 1. The generation of hemolytic activity in a mixture of 2×10^9 *T. congolense*/ml and an equal volume of 2.5% washed sheep erythrocytes.

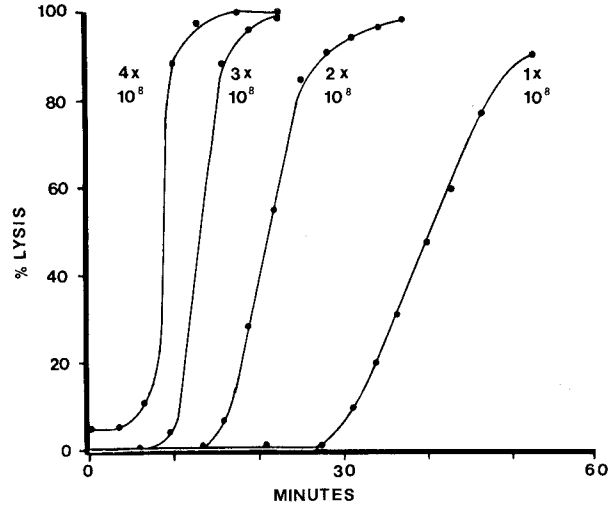

Fig. 2. The effect of dilution of trypanosomes on the rate of hemolysis of a 2.5% suspension of sheep erythrocytes.

Sonicated fresh trypanosomes produced hemolysin at the same time in the same amounts as unsonicated organisms. Hemolysin generated by autolysis for nine hours was capable of lysing an equal volume of 2.5% human, bovine, sheep, horse, mouse, rat or rabbit erythrocytes, in addition mouse peritoneal cells and rabbit buffy coat cells were rapidly killed. The activity of the hemolysin was also shown to be temperature dependant, the T_{50} (time to 50% hemolysis) which was 2 min at 37 °C, increased to 12 min at 30 °C and to > 90 min at 4 °C when tested on sheep erythrocytes. The rate of hemolysis of sheep erythrocytes was also found to be influenced by the relative concentrations of trypanosomes and erythrocytes present in the reaction mixture. Dilution of trypanosomes or an increase in the concentration of erythrocytes resulted in a decline in the hemolytic rate and in a prolongation of the lag period prior to the occurrence of detectable lysis (Figure 2). We suggest that this may indicate a requirement that a critical amount of hemolysin be present prior to cell membrane rupture. The activity of this hemolysin was also calcium dependent being completely inhibited in the presence of 0.005 *M* EDTA. This inhibition could be reversed by the addition of Ca^{++}. Perhaps the most dramatic of the properties of this substance is that all its biological activity i.e. hemolysis of sheep erythrocytes, cytolysis of mouse peritoneal cells and in inflammation following intradermal inoculation into rabbit skin, is retained following incubation at 100 °C in a boiling water bath for 15 min at pH 5.5.

The nature of this trypanosome derived material is unclear. It behaved in enzymic fashion and may possibly be a protease or a phospholipase. Although trypanosome suspensions do possess weak protease activity as measured on Azocoll (Calbiochem), all proteolytic activity was completely destroyed by treatment at 100 °C for 15 min. In addition hemolysis was unaffected by the presence of either trasylol (Boehringer, Canada) 100 units/ml or soy bean trypsin inhibitor (Sigma) used at 0.3 mg/ml, both of which are protease inhibitors.

Although phospholipase activity has not been demonstrated to be present in these hemolytic preparations, many of the activities and properties reported here are similar to those of phospholipase A. Thus phospholipase A is also stable on boiling[10], calcium dependent[11] and hemolytic.

An active phospholipase released, even in small quantities from organisms contained within capillaries, is potentially capable of causing hemolysis and hence anemia[12], platelet aggregation[13] and hence microthrombus formation and local changes in vascular permeability[14] resulting in pericapillary edema. All of these are features of *T. congolense* infection[15]. We therefore suggest that the active material described here may play a significant role in the pathogenesis of disease due to *T. congolense*.

A suspension of an equivalent number of freshly isolated *Trypanosoma lewisi*, a non-pathogenic organism, incubated with erythrocytes at 20 °C produced no significant hemolysis even after 3 days.

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