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# CHANGES IN THE ALLANTOIC FLUID COMPOSITION DURING THE DEVELOPMENT OF THE CHICKEN EMBRYO. INFLUENCE OF SOME FACTORS ON THE VIABILITY OF *TRYPANOSOMA BRUCEI*

by

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*Summary* — Some chemical substances and physical properties of the allantoic fluid from the chicken embryo were followed daily during the normal embryogenesis and after infection with *T. brucei*, in order to check if variations of some among them are liable to influence the viability of the trypanosomes.

The factors studied were : volume and pH of the allantoic fluid, its conductivity, its concentration of proteins, glucose and uric acid concentrations as well as the degree of infestation by the parasites; the acid and alkaline phosphodiesterase activities have also been investigated.

The allantoic fluid was also submitted to gelfiltration.

The respective importance of the studied factors on the viability of the trypanosomes is discussed; it appears that the increase of the phosphodiesterases could be the main causal factor of the disappearance of the trypanosomes from the allantoic fluid during the embryonic development.

## Introduction

The allantoic sac contains a liquid, the composition and volume of which are changing during embryonic development (Fiske and Boyden, 1926; Boyden, 1929; Romanoff *et al.*, 1938; Romanoff, 1960).

Early in the embryonic life the allantoic fluid is derived from the wall of its sac or from the cloaca. After the fourth day urine begins to flow in the allantois. The fluid becomes yellowish at the eleventh day. Before the fourteenth day the fluid reaches its maximum volume and salts of uric acid begin to be deposited in solid form. The fluid diminishes in amount after the fourteenth day of incubation as a result of the absorption of water. The twentieth day the liquid has practically disappeared.

The incubated chicken egg is a well known culture medium for numerous viruses. Biocea (1938) and Longley *et al.* (1939) first cultured trypanosomes on the allantoic membrane, but the embryos died before the tenth day of infection. Independently Hallauer and Kühn (1940) confirmed this method and claimed also that the allantoic fluid provided a good and in certain respects, even a better medium to raise the trypanosomes without killing the embryo. A review has been made in 1943 by Rodhain and van den Berghe of the numerous trypanosome species and strains cultivable on chicken eggs.

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Other authors recently used this technique to elucidate some problems. In 1962 Fagard *et al.* found trypanosomes in chickens, infected during embryonic life in the allantois. It has also been demonstrated by Maillot (1968) that tse-tse flies are able to infect eggs. Goedbloed and Southgate (1969) confirm the universal immunological law that no antibodies against trypanosomes are formed in the infected chicken embryo although fluctuation of the level of parasitemia is apparent.

Bienz (1967 a and b) tried to find out why the trypanosomes have completely disappeared from the inoculated allantois at the sixteenth day of incubation; they populate the blood of the embryo from the fourteenth day on within 24-48 hours. He finds that this was due to a modification of the allantoic fluid and not to exhaustion of the culture medium by the trypanosomes. Bienz further determined the pH of the allantoic fluid and stated a correlation between the pH decrease beneath pH 6.5 and the disappearance of the trypanosomes. He was able to exclude the effect of toxic metabolites of the trypanosomes. Throughout the experiments that we are reporting now, we tried to complete the work of Bienz.

## Materials and methods

In order to make the results comparable, a strain of *Trypanosoma brucei* (EATRO 1125) cultivated on mice was used (*). Trypanosomized mouse blood was inoculated in the allantoic sac of ten days incubated chicken embryos. The fluid was totally removed by direct punction from the seventh to the seventeenth day of incubation. Every day at least four allantoic fluids were examined. When the volumes of the allantois were too low, four pools were made, to obtain a sufficient volume ($\pm$ 5 ml).

The presence of parasites was followed by microscopic examination.

The pH was determined with a Metrohn pH meter Type E 396 B.

Uric acid concentration was estimated by taking the spectra of the purified uric acid fractions at different pH values, using a Beckman DB$^{R}$ (double-beam) spectrophotometer.

Conductivity studies were performed with a Radiometer Conductivity meter Type CDM2e.

The glucose concentrations were estimated with the enzymatic method of Schmidt (1963). The reaction was calibrated with control solutions and concentrations of 0.04 mg/ml could be detected.

The protein content of the allantoic fluid was determined in the purified protein fraction, after gel filtration on SG-75, by the spectrophotometric method of Warburg and Christian (1941) and was calculated with the correction formule of Layne (1957).

The purification technique consisted of gel filtration on Sephadex G-10 and Sephadex G-75. An excellent monograph of this technique is given by Determan (1967). Columns with a diameter of 14 mm and a lenght of 60 mm were used.

Alkaline and acid phosphodiesterase activities were determined and followed during embryogenesis in the allantoic fluid. The determination of these enzymes, described and studied some years ago in fertilized and

(*) Thanks go to Prof. Dr. Mortelmans who supplied the allantoic fluids needed in this study.

unfertilized chicken eggs, is given in earlier publications (Moors and Stockx 1966, 1968, 1971).

## Results

### *Presence of trypanosomes*

Trypanosomes were found in fluctuating numbers in the allantoic fluid from the inoculation till the fifteenth day of incubation in the eggs examined every day after infection. The morphology of the trypanosomes was not entirely ascertained, but in agreement with earlier work, they were not polymorph.

### *pH*

The pH of the allantoic fluid from infected and normal embryos during embryogenesis are identical. These results are in agreement with the pH measurements of Bienz (1967). The pH fall on the fourteenth day is clear (figure 1 a).

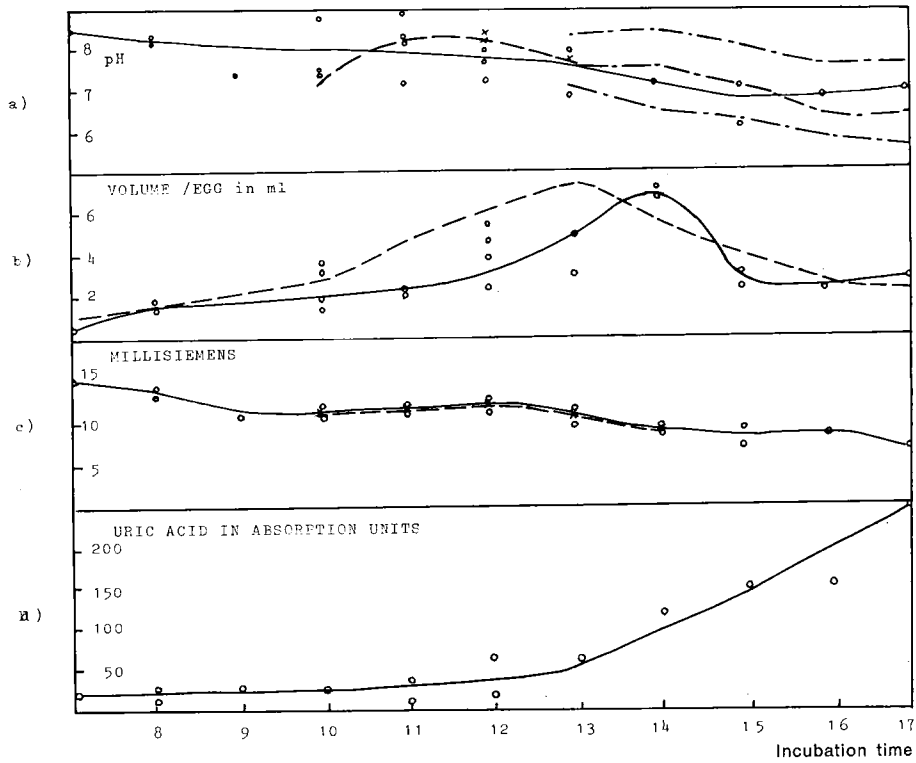


Figure 1

a : pH of the allantoic fluid/incubation time
uninfected eggs O————
trypanosomized eggs — × — — —
values of Bienz (1967) - — - — - —

b : Volume of the allantoic fluid/incubation time
of our eggs ————
values of Romanoff and Hayward (1943) - -

c : Conductivity in millisiemens/incubation time
uninfected eggs ————
trypanosomized eggs — — — — — — — —

d : The uric acid level in the allantois during embryo-genesis in absorption units at 292 nm

## *Volume*

The volume of allantoic fluid in normal and infected embryos is almost similar. The volume curve found during embryogenesis is compared with one determined by Romanoff and Hayward (1943) for the allantoic fluid of the Leghorn chicken embryo (figure 1 b).

The small differences between the volume and pH curves of our experiments and those given by other authors are probably due to an incubation temperature of maximum 1 °C above or below 37.5 °C.

## *Uric acid and Urates*

Figure 2 gives the spectra of not entirely purified uric acid taken at pH 2, 7 and 12 after gel filtration of the allantois from 14 days incubated eggs. The deposited urates were dissolved as good as possible by dilution and taken into account. The uric acid level in the allantois during embryogenesis is given in figure 1 d in absorption units at $\lambda_{max}$ : the wave length of maximal absorption 292 nm; the molarity may be estimated when comparing the absorption units with the molar absorption coefficient $\varepsilon_{max}$ of uric acid, which is $11.6 \times 10^3$ as determined by Green and Mazur (1957) and by Volkin and Cohn (1954).

During the period that the trypanosomes disappear from the thirteenth day on, the uric acid concentration considerably increases up to a concentration of at least 4 mg/ml.

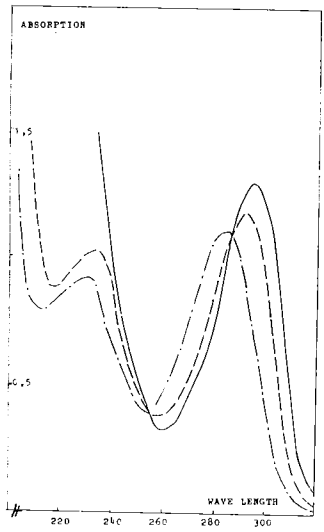


Figure 2

The spectra of not entirely purified uric acid 1/100 diluted taken at pH 2 — · — · —
pH 7 — — —
pH 12 ———
after gel filtration of the allantois from 14 days incubated eggs.

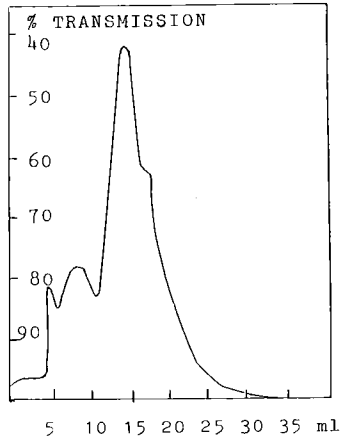


Figure 3

Gel filtration on a Sephadex G-10 column (l = 60 mm, Ø = 14 mm) of the allantoic fluid from 13 days incubated eggs.

Spectrophotometric studies in the visible region indicate that the yellow colour which appears in the fluid is probably the result of an increase in uric acid concentration.

### *Conductivity*

The seventh day of incubation a conductivity of 15 millisiemens is measured. The conductivity decreases till the ninth day, remains stable (more or less) between eleven and nine millisiemens during the fixe following days whereafter a slight decrease is observed (figure 1 c). There is no difference in conductivity caused by the infection.

### *Glucose*

Glucose was never detected in the allantoic fluid of infected and non infected eggs. Therefore the glucose content must be lower than 0.04 mg/ml.

### *Proteins*

The protein content varies approximately 2 mg/ml during the whole observation time. Differences in protein concentration are found between the allantoic fluids of different eggs at the same incubation day. Moreover infected eggs could not be distinguished from uninfected ones by means of protein concentration measurements. During the investigated incubation time the maximum protein content ($\pm$ 4 mg/ml) is found on the fifteenth day.

### *Gelfiltration*

Allantoic fluid in 0.4 ml amounts, submitted to gel filtration on Sephadex G-75 between the 7th and 17th incubation day, gives always two peaks; the first peak contains all the macromolecules e.g. the proteins, the second contains only molecules with a low molecular weight. It is also possible to separate the allantoic fluid on Sephadex G-10 in four distinct fractions (figure 3), the third peak containing practically pure uric acid.

### *Acid phosphodiesterase activity*

The acid phosphodiesterase activity of the allantoic fluid is not influenced by the infection with trypanosomes. As shown in figure 4 b which represents the enzymatic activity levels during the incubation period, great variations are not observed.

The concentration is very low from the seventh untill the eleventh day and increases tenfold between the eleventh and the sixteenth day. Figure 4 a gives the pH optimum curve of the acid phosphodiesterase of the allantois. The pH optimum of 4.7 $\pm$ 0.1 is nearly the same of that of acid phosphodiesterase of the chicken egg.

Figure 4 a gives also the pH optimum curve of the alkaline diesterase of the allantois. A pH optimum of 8.0 ± 0.1 is also found for egg yolk and white. The alkaline phosphodiesterase activity is always higher in the allantois during embryogenesis than the acid diesterase activity. Till the fourteenth day the activity is low. The fifteenth day a very sharp increase is observed (figure 4 b). The increase cannot be due to the disruption of trypanosomes which have a low alkaline diesterase activity but may be the cause why trypanosomes disappear. The activity/concentration curve is practically a straight line (figure 4 c).

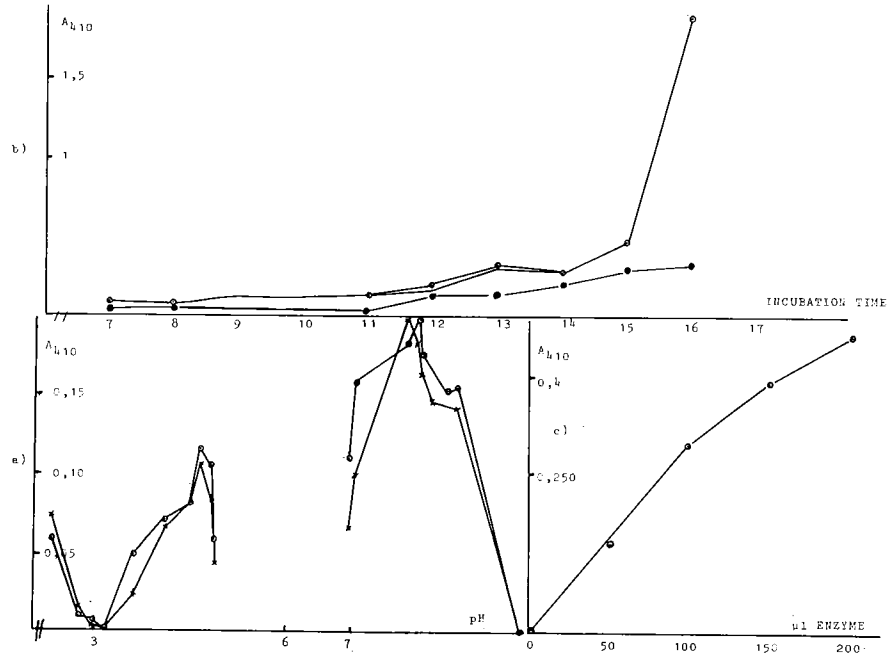


Figure 4

a : The pH optima curves of the acid and the alkaline phosphodiesterase of the allantoic fluid of the eleventh incubation day : incubation time : 200 min.
incubation temperature : 37.50 °C.
incubation mixture : 2 ml.
substrate 0.004 M in 0.1 M citrate or acetate or tris or carbonate + 0.2 ml enzyme.

b : Diesterase activities of the allantois/incubation time
Acid diesterase (measured at 0.2 M acetate pH 4.9 ●————
Alkaline diesterase (measured at 0.2 M tris ph 8.5 O————
incubation time 200 min.; incubation temperature 37.5 °C.

c : Alkaline diesterase activity/enzyme concentration of the allantoic fluid of the fifteenth incubation day.
incubation time 200 min.
incubation temperature 37.5 °C.
incubation mixture 2 ml substrate 0.005 M
0.2 M tris pH 8.5
+ 0.2 ml enzyme.

## Discussion

As a culture medium for trypanosomes, the allantoic fluid is comparable to the faecal material of a hen with the restriction that a great part of biological active products, e.g. enzymes catalysing hydrolysis, are not broken down after use. This explains already the absence of trypanosomes. The absence of glucose in the allantoic fluid also makes the medium very unsuitable for the growth of trypanosomes if the strains used need glucose, as already discussed by von Brand (1952) and by Ryley (1956).

The pH decrease beneath pH 6.5 may play a role in the disappearance of trypanosomes, but other factors are also to be considered e.g. : the increase of acid diesterase, the decrease in conductivity after the eleventh day; the increase of uric acid from the thirteenth day on; the absorption of water and salts by the embryo from the fourteenth day, the appearance of IgG globulins of maternal origin from the fourteenth day on in the allantoic fluid (Kramer and Cho, 1970) and finally the tenfold increase of alkaline phosphodiesterase activity between the fourteenth and the fifteenth day.

Particularly the increase of alkaline phosphodiesterase activity seems of interest, not only because the activity reaches, in the period that the trypanosomes disappear, the highest levels but also because of the possible nocuous activity of a phosphodiesterase.

Although the chemical, *in vitro*, specificity of our diesterase is not entirely elucidated, a « non-specific » phosphodiesterase catalyses the cleavage of one of the phosphate ester bonds in a phosphodiester linkage in simple phosphodiesters as well as in polynucleotides of the ribose and deoxyribose types. Snake venom phosphodiesterase e.g. hydrolyses DNA and RNA as an exonuclease and acts on oligonucleotides as 5'nucleotidase to liberate 5'nucleotides, it cleaves also the pyrophosphate bonds of ATP, ADP, UTP, etc., to yield the corresponding nucleoside 5'phosphates (Laskowski *et al.*, 1957). A similar phosphodiesterase activity is found in some egg lipid preparations (Moors, 1969).

The physiological in vivo activity of diesterases is less well known. Our results with the alkaline diesterase of cavia serum and the results of Cochrane *et al.* (1970) and Birdsey *et al.* (1971) with fractions of snake venom suggest that there is an interaction with the complement system.

According to Laveran and Mesnil (1912) and Laveran (1904) snake venom and « lecithides » kill trypanosomes *in vitro*. This trypanocide action depends on temperature which suggests enzyme action. Further details of the alkaline diesterase action on trypanosomes are in investigation.

*Samenvatting* — **Wijzigingen in de allantois-vocht samenstelling tijdens de ontwikkeling van het kippenembryo. Invloed van sommige faktoren op de leefbaarheid van** *T. brucei*.

Een reeks chemische en fysische parameters van het allantois-vocht van kippenembryo's werden gevolgd in funktie van de embryogenesis en van de besmetting met *T. brucei*. Sommige gekende faktoren werden ter kontrole opnieuw onderzocht zoals infestatiegraad, volume en pH van het allantoisvocht. Ook nieuwe parameters werden bestudeerd : het urinezuurgehalte, de conductiviteit, het proteïnegehalte en de glucoseconcentratie. Het allantoisvocht werd tevens aan gelfiltratie onderworpen. De zure en alkalische diesterase aktiviteiten werden gevolgd. Enkele faktoren die de trypanosomen zouden kunnen verdrijven uit de allantoiszak werden aangegeven. De stijging tijdens de embryogenese van de diesterases zou wel eens de voornaamste

oorzaak kunnen zijn van het verdwijnen der trypanosomen uit de allantoiszak. Deze en andere hypothesen worden aan een grondige discussie onderworpen.

*Résumé* — **Variations dans la composition du liquide allantoïdien pendant le développement de l'embryon de poulet. Influence de certains facteurs sur la survie de** *T. brucei*.

Plusieurs paramètres physiques, chimiques et physiologiques du liquide allantoïdien de l'embryon de poulet ont été mesurés quotidiennement au cours de l'embryogenèse normale, ainsi qu'au cours de l'infection par *T. brucei*, notamment en vue de vérifier si des variations de certains d'entre eux sont susceptibles d'influencer la survie de ces trypanosomes.

Les facteurs suivants furent étudiés : volume et pH du liquide allantoïdien, sa conductivité, les taux de protéïnes, de glucose et d'acide urique, ainsi que le degré d'infestation par les parasites; l'évolution de l'activité des diestérases acide et alcaline a également été suivie.

Le liquide allantoïdien a été chromatographié sur Sephadex G-10 et G-75.

L'importance respective des facteurs étudiés sur la survie des trypanosomes est discutée; il en ressort que l'élévation des phosphodiestérases pendant le développement pourrait être la cause principale de la disparition des trypanosomes du liquide allantoïdien au cours du développement embryonnaire.

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