The effects of generally used anticoagulants on the haemolysis of fish erythrocytes

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Abstract

Ethylenediamine tetra-acetate (EDTA), ammonium potassium oxalate (APO), tri-sodium citrate (TSC) and heparin were evaluated for their suitability as anticoagulants for the blood of *Oreochromis mossambicus* and *Cyprinus carpio*. It was found that EDTA, APO and TSC were unsuitable due to their haemolytic effects. Heparin was found to be the anticoagulant of choice.

Introduction

Haematological profiles are increasingly being used as diagnostic aids to determine the health status of fish (Blaxhall, 1972; Smit and Hattingh, 1978). However, many factors can influence the results in such a way that it is difficult to establish proper base values for the various parameters to be determined (Van Vuren and Hattingh, 1978). It is therefore essential to obtain samples representing the true blood physiological status of the experimental animals.

Investigations have shown that heparin, citrate and oxalates are commonly used as anticoagulants for fish blood (Blaxhall 1973; Smit *et al.*, 1977; Barham *et al.*, 1979; Smit, 1980). Results indicate that variations occur in the various blood parameters investigated, especially haemoglobin concentration, erythrocyte counts and erythrocyte sedimentation rate (ESR) (Blaxhall, 1972). It was also reported that oxalates and ethylenediamine tetra-acetate (EDTA) cause haemolysis of fish erythrocytes (Smit and Hattingh, 1980), rendering the blood sample unsuitable for analysis.

In view of the above, this study was undertaken to observe, under controlled and standard conditions, the effects of time and various concentrations of generally used anticoagulants on the haemolysis of fish erythrocytes. It was done because this phenomenon could be responsible for variations in the erythrocyte associated parameters as well as other haematological and blood chemistry values. The degree of haemolysis was determined by measuring the optical density of plasma.

Materials and methods

Adult species of both sexes of *Cyprinus carpio* and *Oreochromis mossambicus* were obtained from the Fisheries Research Station at Marble Hall (Transvaal, South Africa). The fish were acclimated for at least 3 months in large aquaria at a temperature of 19 ° ± 1 °C. Seven days prior to experimentation, the fish (20 fish of both sexes and species) were transferred to smaller laboratory aquaria of 45 ℓ capacity, under similar environmental conditions in order to minimize possible stress effects (Pieterse *et al.*, 1981).

Anticoagulant concentrations were prepared in such a way that 2 mg, 4 mg and 6 mg EDTA, TSC and APO per ml blood were present after mixing with 3 ml blood (Raphael, 1976). Only 6 mg heparin per ml blood was used to act as control. The reason for this is that all the 2, 4 and 6 mg/ℓ concentrations of heparin used produced no haemolysis in the erythrocytes of both fish species and, therefore, only the highest heparin concentration was used as a conrol. Ten fish of each species were used for each concentration of anticoagulant. The fish were anaesthetized with 100 mg/ℓ neutralized benzocaine hydrochloride (Ferreira *et al.*, 1979) and 3 ml blood was collected by cardiac puncture (Van Vliet, 1981) into syringes containing the desired amount of anticoagulant. The blood was carefully mixed by rolling the syringe between the hands and thereafter 0,5 mℓ aliquots were placed in six glass test tubes and left at room temperature until examined. For identification the test tubes were labelled as follows: Time 0 min (T = 0; T = 30; T = 60; T = 120 and T = 180 min). The optical density of sample T = 0 was used as blank reading. Since haemolysis occurred very rapidly with APO, the time lapse between erythrocyte counts was reduced as follows: T = 0; T = 10; T = 30 min. Immediately after collection, erythrocyte counts were carried out on blood sample T = 0, using an improved Neubauer counting chamber (Dacie and Lewis, 1963). During the counting procedure only erythrocytes that appeared undamaged were evaluated. The free-lying nuclei of the lysed erythrocytes were not counted since they represented the number of lysed cells. As soon as the blood dilution for erythrocyte counts was made, the remaining blood was centrifuged at 2 000 r/min for 20 min and the plasma was transferred to a clean test tube. The procedure was followed for each sample.

The plasma was then diluted 1:10 by mixing 0,1 ml plasma with 0,9 ml distilled water. The optical density of the dilution was then read at 545 nm on a Spectronic 70 spectrophotometer.

Results

As already said, all the concentrations of heparin used in the experiments produced no haemolysis in the erythrocytes and the highest concentration heparin (6 mg/ml) was used as a control. The other anticoagulants caused various degrees of haemolysis of the erythrocytes of both fish species (Figs. 1 and 2). However, the intensity of haemolysis was markedly less with the lower concentrations of anticoagulants, except in the case of APO which caused immediate haemolysis of 80% of the erythrocytes being complete in less than 15 min (Figs. 3 and 4). EDTA at 2 mg/ml blood produced no haemolysis in *C. carpio*. At 4 mg and 6 mg/ml EDTA haemolysis was produced in both fish species within 20 min (Figs. 5 and 6). TSC at 2 mg and 4 mg/ml blood produced little or no haemolysis in *O. mossambicus* after 180 min.

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Received 28 September 1984.

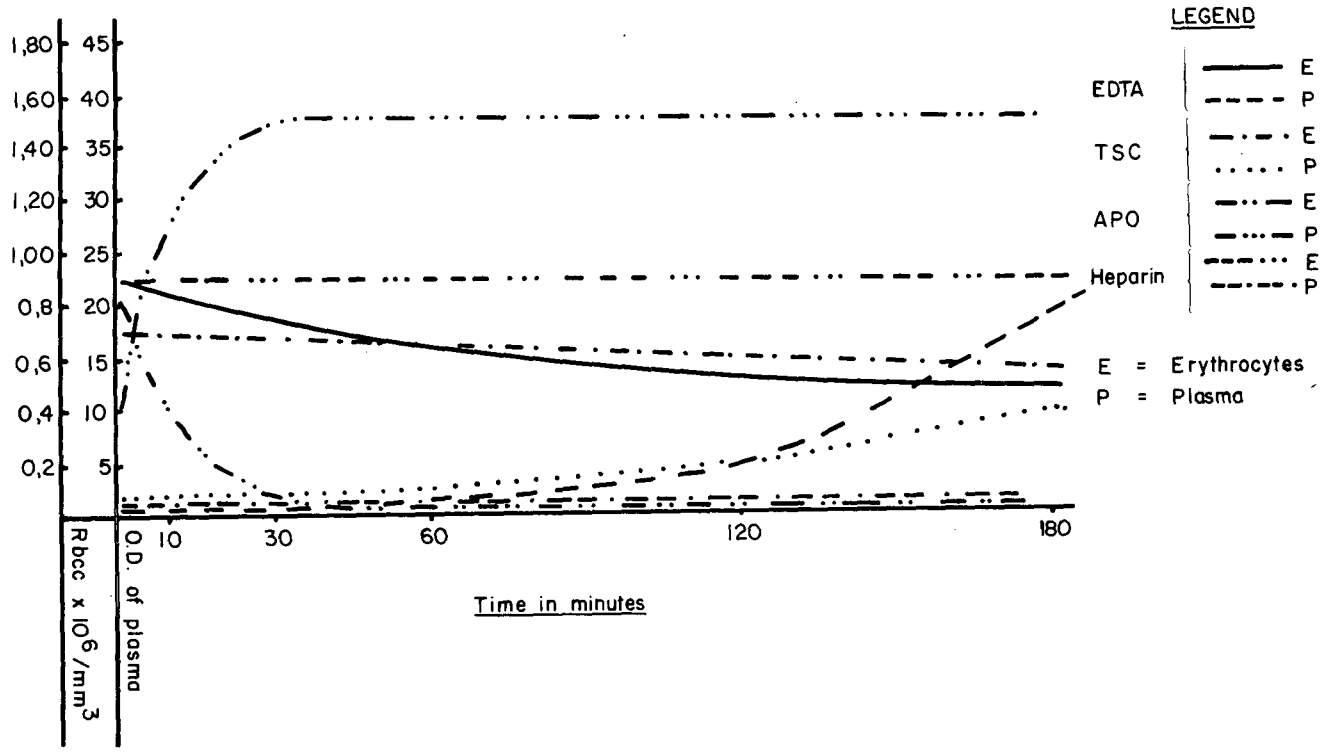

Figure 1
Effect of all concentrations of heparin, APO, EDTA and TSC on haemolysis of erythrocytes in Cyprinus carpio.

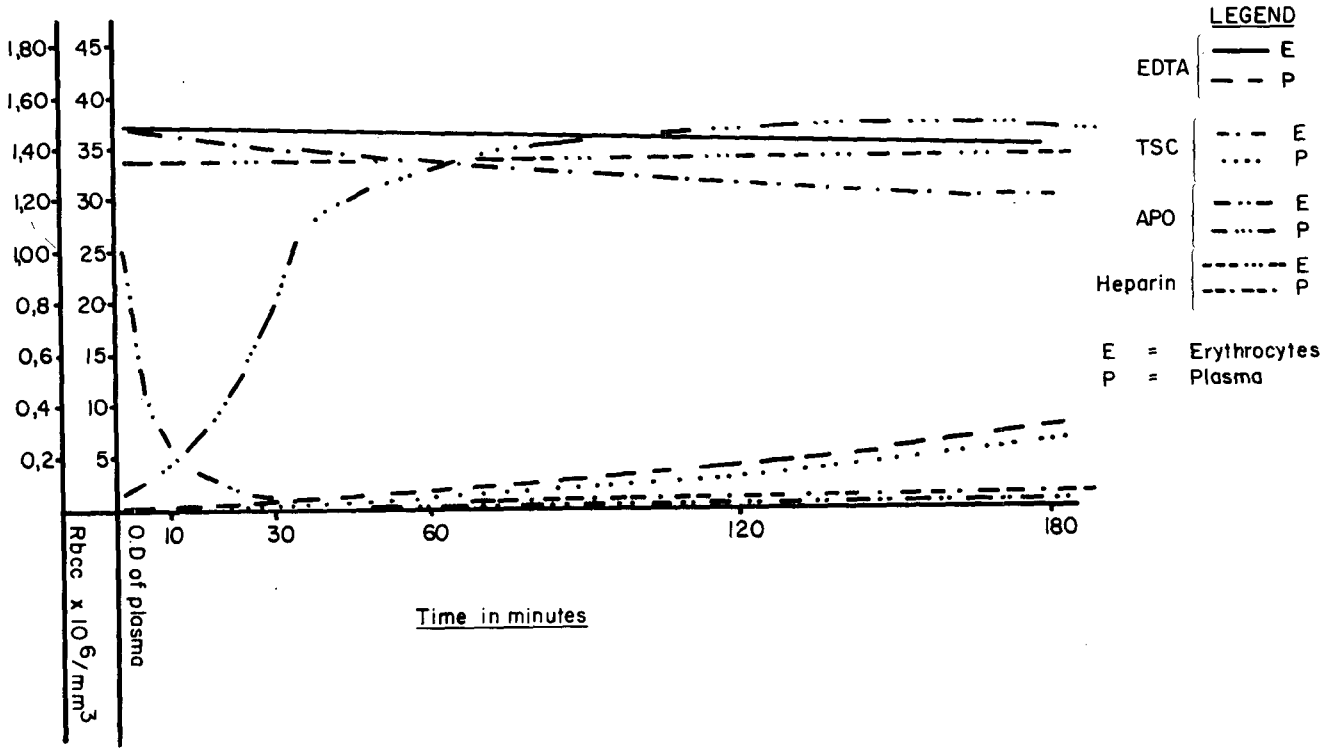

Figure 2
Effect of all concentrations of heparin, APO, EDTA and TSC on haemolysis of erythrocytes in Sarotherodon mossambicus.

However, 6 mg/ml caused severe haemolysis after 10 min. In *C. carpio*, 2 mg TSC/ml blood produced only slight haemolysis after 180 min. With 4 mg/ml TSC haemolysis occurred after 60 min, whereas 6 mg/ml resulted in almost immediate haemolysis (Figs. 7 and 8).

Discussion

Results of previous authors did not always clearly indicate whether fish were under stress during and just prior to sampling, or whether the blood samples were collected without trauma

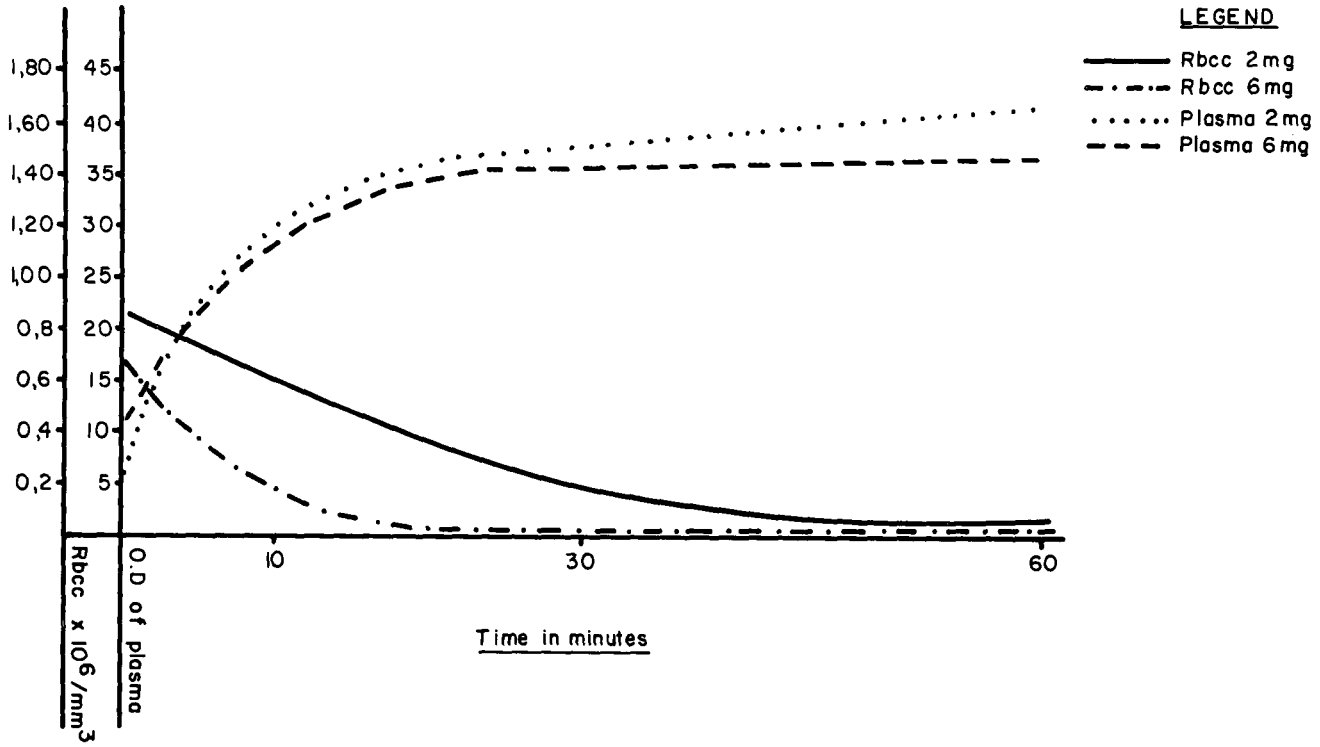

Figure 3
Effect of various concentration APO on Haemolysis of erythrocytes in Cyprinus carpio with atraumatic blood.

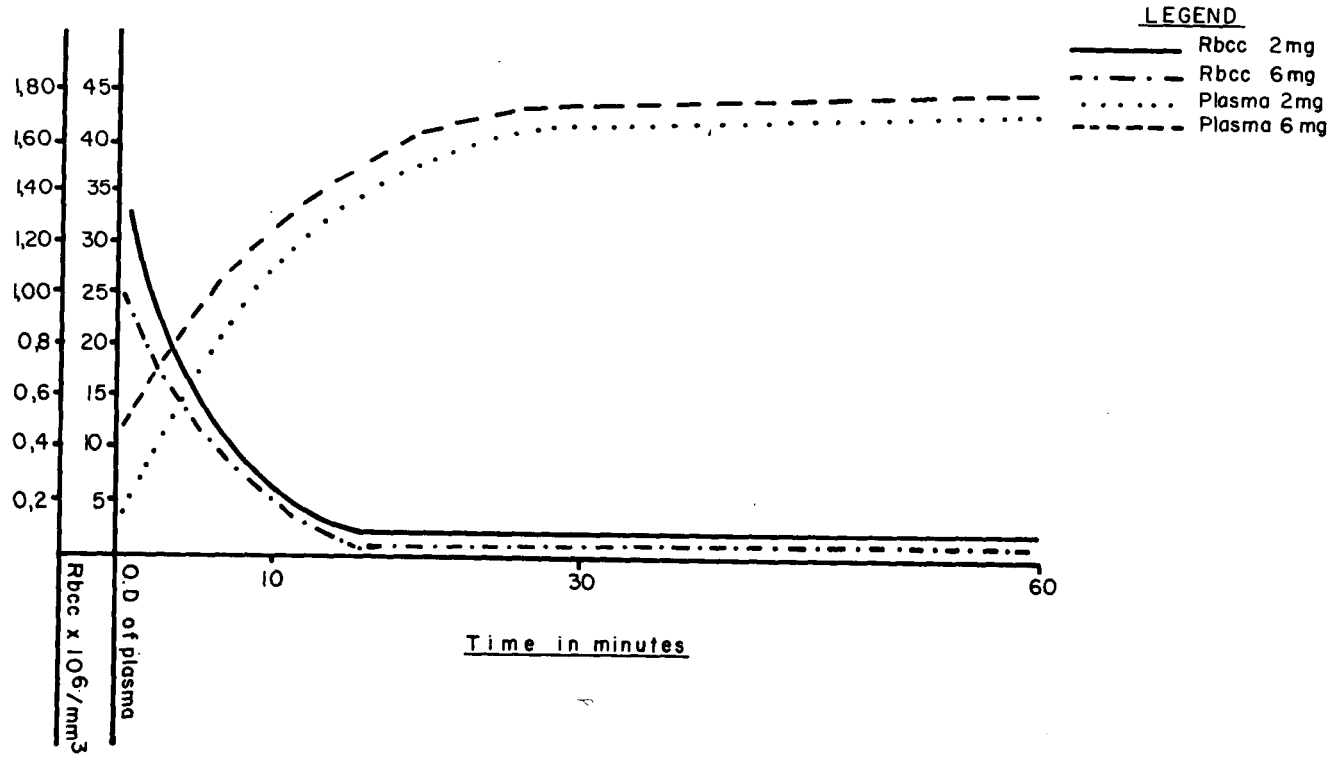

Figure 4
Effect of various concentrations APO on haemolysis of erythrocytes in Sarotherodon mossambicus with atraumatic blood.

(Smit and Schoonbee, 1976; Smit *et al.*, 1977; Smit and Hattingh, 1978). Therefore, care was taken that the fish were kept very calm in order to minimize possible stress effects. Under these conditions it was shown that APO, EDTA and TSC had definite haemolysing effects on the blood samples.

APO caused severe haemolysis of fish erythrocytes within a few minutes after mixing with the blood, whereas TSC and EDTA had haemolysing effects, varying from partial to total haemolysis, after being stored for 10 min. APO, TSC and EDTA are artificial anticoagulants which prevent coagulation by

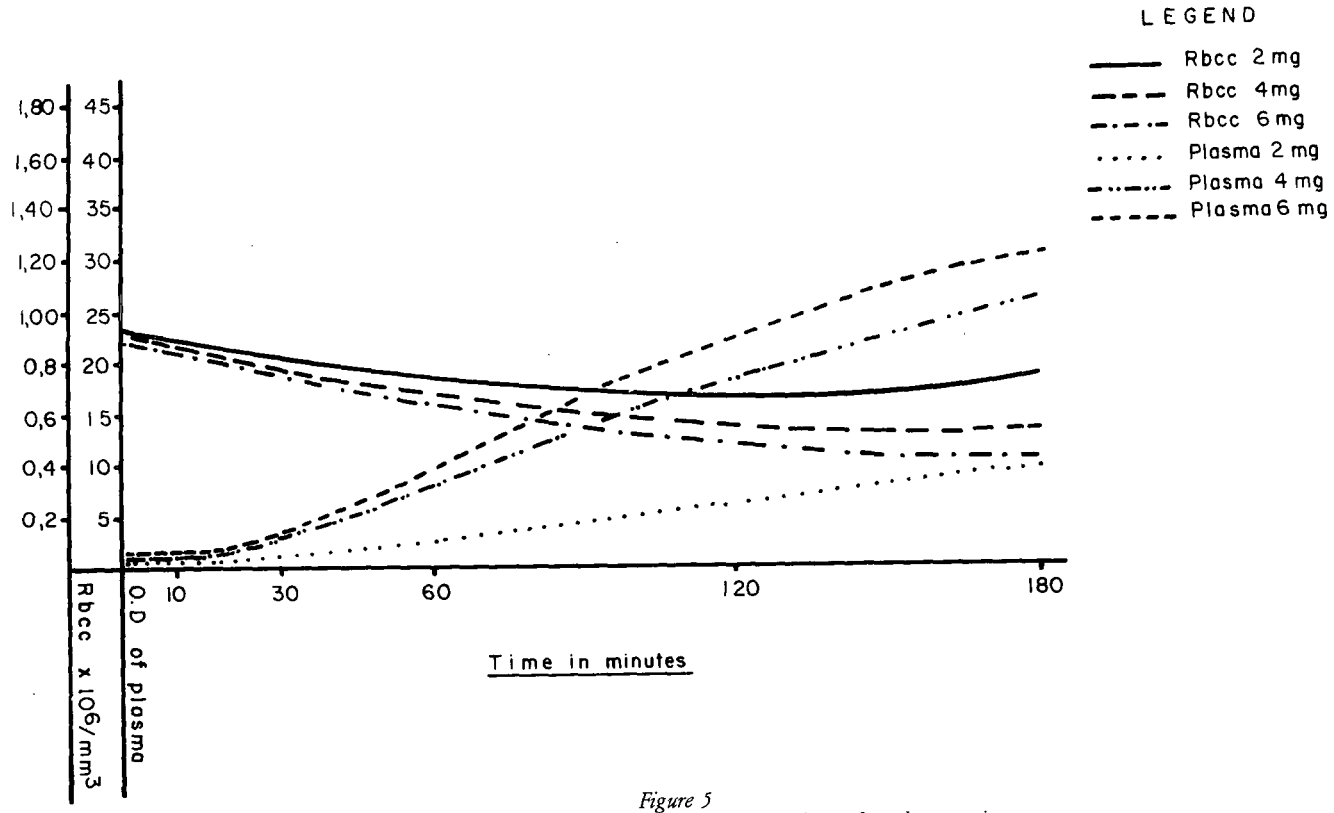

Figure 5
Effect of various concentrations EDTA on haemolysis of erythrocytes in Cyprinus carpio with atraumatic blood.

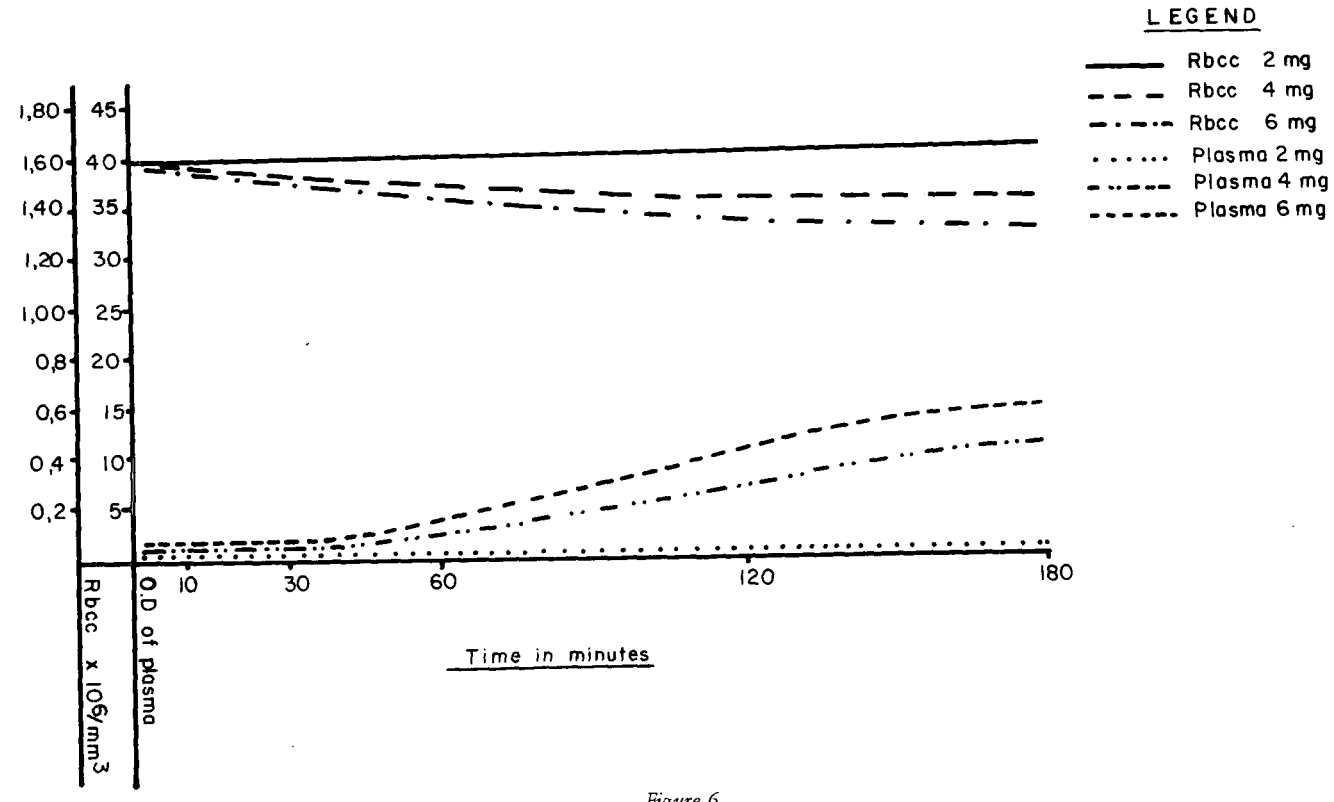

Figure 6
Effect of various concentrations EDTA on haemolysis of erythrocytes in Sarotherodon mossambicus with atraumatic blood.

precipitating calcium as insoluble oxalate or bound in an unionized form (Biggs, 1976). Since calcium is also present in the membranes, it is possible that the precipitation of insoluble oxalate and formation of unionized calcium may result in rupture of the erythrocyte membranes, rather than increased permeability of haemoglobin through the erythrocyte membrane.

Microscopical examination of the blood sample during the enumeration of the erythrocytes showed that erythrocyte nuclei were present with the cell membrane remnants clearly distinguished from intact cells. Differences observed in the

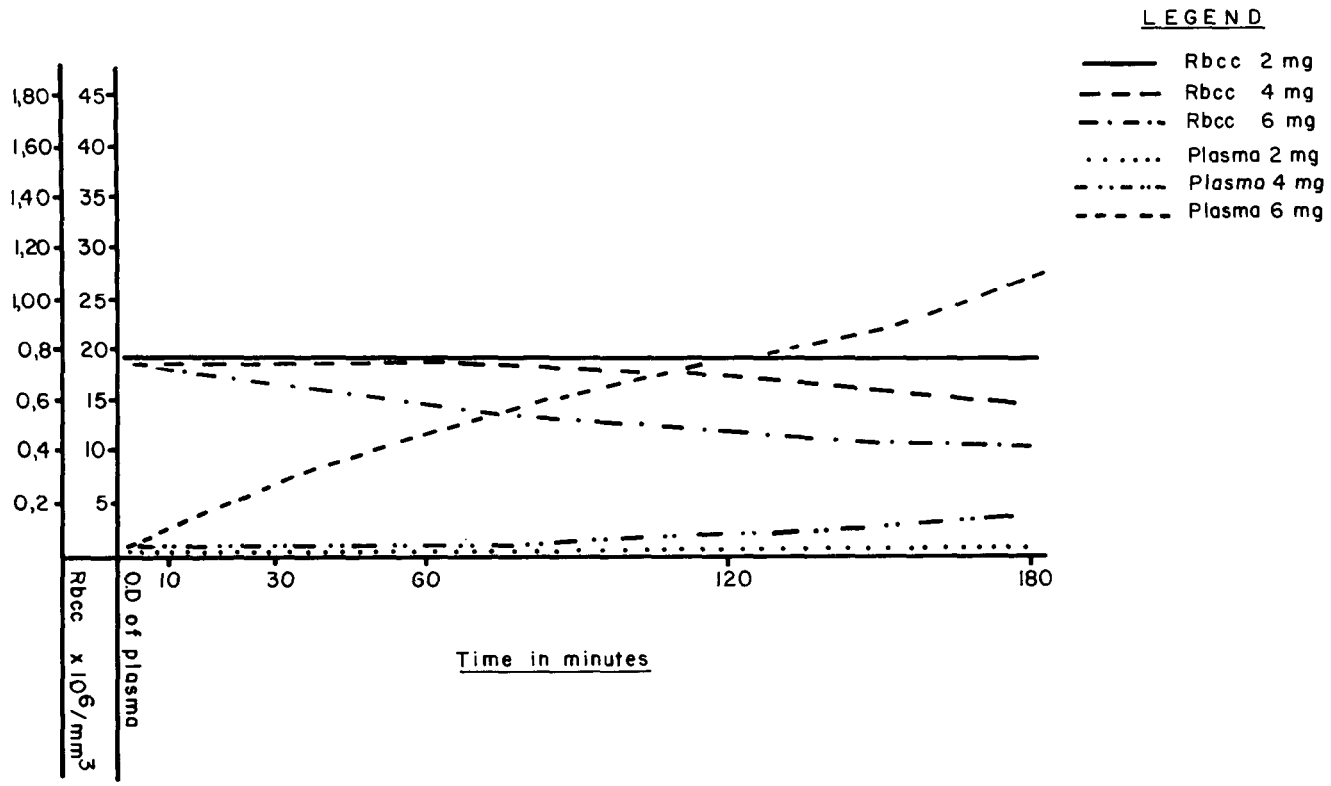

Figure 7
Effect of various concentrations of TSC on haemolysis of erythrocytes in Cyprinus carpio with atraumatic blood.

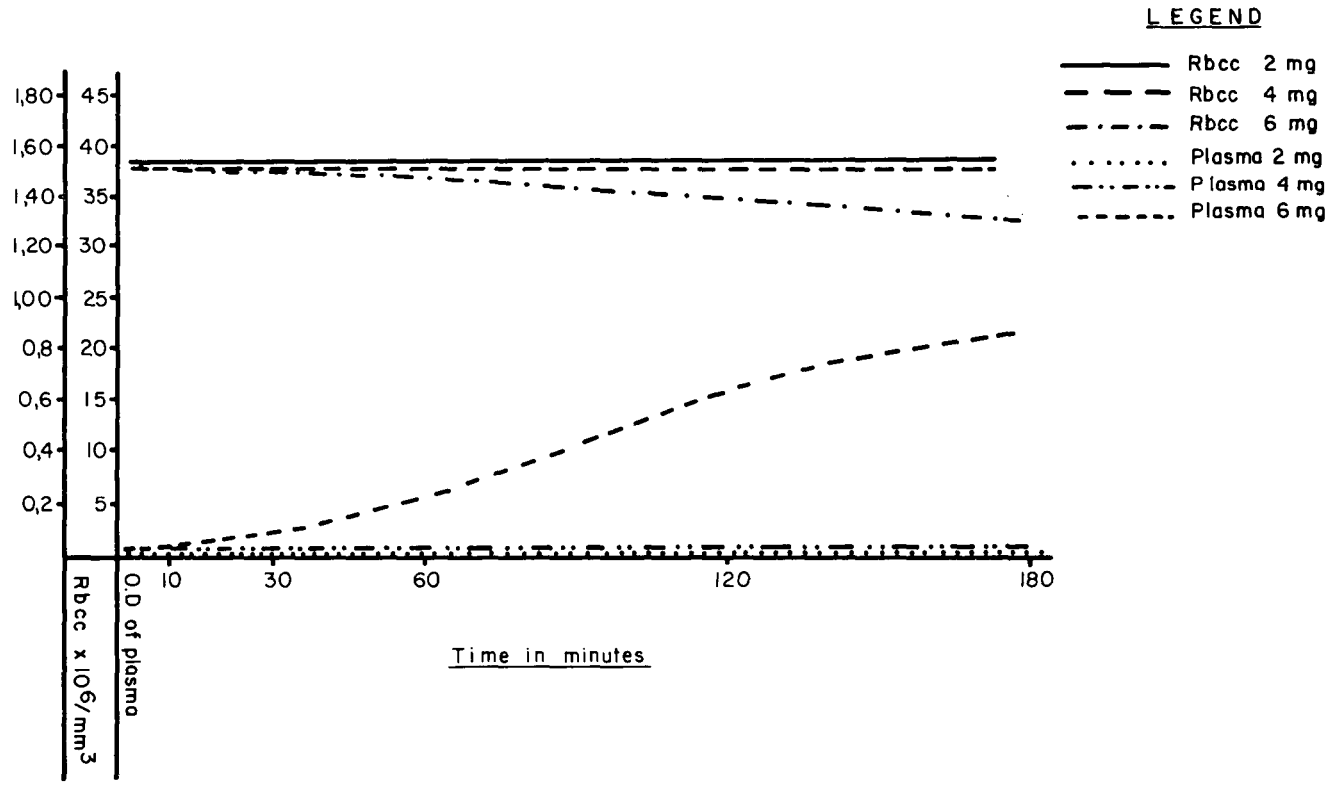

Figure 8
Effect of various concentrations of TSC on haemolysis of erythrocytes in Sarotherodon mossambicus with atraumatic blood.

haemolysing effect of all these anticoagulants over a period of three hours in both species, suggest that the artificial anticoagulants have different haemolysing rates.

All concentrations of EDTA used resulted in partial haemolysis of carp erythrocytes after a few minutes, which increased over a period of three hours, being less severe at the lower concentrations. The haemolysis is less severe in *O. mossambicus* than in *C. carpio*, possibly due to difference in the Hb contents of the erythrocytes, being higher in *C. carpio*. Similar effects were obtained in *C. carpio* with TSC. However, in *O. mossambicus*,

haemolysis occurred only with 6 mg/ml TSC.

From the results it is evident that the erythrocytes of *O. mossambicus* can tolerate haemolytic influence better than those of *C. carpio*. Van Vliet (1981) found that blood coagulation times were reduced after a certain period of time in *C. carpio*, but not in *O. mossambicus*. This suggests the presence of certain clot promoting factors in the erythrocytes which were released during haemolysis, since it was found that *C. carpio* erythrocytes lysed more rapidly and intensively than those of *O. mossambicus*. It is possible that the erythrocyte cytoplasm contains potent thromboplastic substances capable of promoting intrinsic thromboplastin generation and thrombin formation, being more pronounced in *C. carpio* (Van Vliet, 1981).

The haemolytic effect of the various anticoagulants should therefore be taken into consideration when fish blood is investigated, since erythrocyte contents may affect the results considerably.

Heparin seems to be the most suitable anticoagulant for use with the blood of these fish. This is true because no haemolysis is caused when this anticoagulant is employed, providing that all other stress factors, before, during and after blood sampling are minimized. Furthermore, when blood is not obtained at the first effort, the sample should be regarded as "traumatic" since it could possibly be contaminated with tissue fluid.

The present results agree with the findings of Smit *et al.* (1977) and Smit and Hattingh (1980) but they strongly emphasize the fact that the sampling of the blood is of the utmost importance. It is further evident that the chemical nature of the anticoagulant cannot be regarded as the only factor determining the suitability of a specific anticoagulant for use with fish blood.

Acknowledgements

The authors wish to thank the Research Committee of the Rand Afrikaans University and Pretoria Technikon for financial support. The supply of fish by the Fisheries Research Station at Marble Hall is highly appreciated.

References

BARHAM, W.T., SMIT, G.L. and SCHOONBEE, H.J. (1979) The effect of heparin concentration on certain blood parameters of the rainbow trout *Salmo gairdneri* Richardson. *Comp. Biochem. Physiol.* **63c** 369-371.

BIGGS, R. (1976) Human coagulation haemostasis and thrombosis. Blackwell Scientific Publications, Oxford, London, Edenburgh, Melbourne.

BLAXHALL, P.C. (1972) The haematological assessment of the health of freshwater fish: A review of selected literature. *J. Fish Biol.* **4** 593-604.

BLAXHALL, P.C. (1973) Error in haematocrit produced by inadequate concentrations of ethylenediamine tetra-acetate. *J. Fish Biol.* **5** 767-769.

DACIE, J.V. and LEWIS, S.M. (1963) Practical haematology 3rd Ed. pp. 18-45, London, Churchill Livingstone.

FERREIRA, J.T., SMIT, G.L., SCHOONBEE, H.J. and HOLZAPFEL, C.W. (1979) Comparison of anaesthetic potency of benzocaine hydrochloride and MS-222 in two freshwater fish species. *Progve. Fish. Cult.* **41** 161-163.

PIETERSE, J.J., SMIT, G.L., VAN VLIET, K., SCHOONBEE, H.J. and HATTINGH, J. (1981) Observations on the blood of the Chinese silver carp *Hypophthalmichthys molitrix* (Valenciennes). *J. Fish Biol.* **18** 455-459.

RAPHAEL, S.S. (Ed.) (1976) *Lynch's Medical Laboratory Technology* Vol. II, 3rd. Ed. London: W.B. Saunders Company.

SMIT, G.L. (1980) The effect of Tricaine-methane sulphonate and generally used anticoagulants on fish haematology. Ph.D. thesis, Witwatersrand University.

SMIT, G.L. and HATTINGH, J. (1978) The effect of respiratory stress on carp haemoglobin. *Comp. Biochem. Physiol.* **59A** 369-374.

SMIT, G.L. and HATTINGH, J. (1980) Haematological assessment of generally used freshwater fish anticoagulants. *J. Fish. Biol.* **17** 337-341.

SMIT, G.L., HATTINGH, J. and SCHOONBEE, H.J. (1977) Observations on some effects of disodium ethylenediamine tetra-acetate and heparin on fish blood. *Comp. Biochem. Physiol.* **57C** 35-38.

SMIT, G.L. and SCHOONBEE, H.J. (1976) Problems encountered with EDTA as an anticoagulant for fish blood. *S.A. Jour. Sci.* **72** 380.

VAN VLIET, K.J. (1981) Blood coagulation and the effects of generally used anticoagulants on the haematology and blood chemistry of *S. mossambicus* and *C. carpio*. M.I. Biol. thesis, Institute of Biology, London.

VAN VUREN, J.H.J. and HATTINGH, J. (1978) A seasonal study of the haematology of wild freshwater fish. *J. Fish Biol.* **13** 305-313.