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## THE EFFECT OF ACETYLPROMAZINE MEDICATION ON RED BLOOD CELL METABOLISM IN THE HORSE

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### Résumé

INFLUENCE DU TRAITEMENT PAR L'ACEPROMAZINE SUR LE METABOLISME DU GLOBULE ROUGE CHEZ LE CHEVAL. — L'emploi répété des tranquillisants et neuroleptiques au cours des compétitions des sports équestres nous a conduit à étudier les effets de l'acépromazine sur l'activité musculaire du cheval de sport. Lors de précédents travaux (Courtot *et al.*, 1975) nous avons conclu à :

- une éventuelle action toxique de ce composé au niveau musculaire, traduite par une augmentation du taux de créatine phosphokinase sérique ;
- l'apparition d'une dépression respiratoire.

Ces observations nous ont amené à poursuivre ces travaux afin de mieux connaître les effets de l'acépromazine au niveau :

- du métabolisme cellulaire ;
- de l'électrogénèse cardiaque.

Le but du présent travail est d'étudier les effets de l'acépromazine au niveau du métabolisme du globule rouge et plus particulièrement :

- du taux globulaire de 2-3 diphosphoglycérate (2-3 DPG) ;
- de l'activité de la glucose 6 phosphate déshydrogénase (G6PD) érythrocytaire ;
- du taux de glutathion réduit (GSH).

L'évolution de ces variables est étudiée avant et après un effort standardisé. L'acépromazine est injectée à dose faible (0,02 mg/kg) une heure avant le début de l'effort.

L'analyse de nos résultats permet de mettre en évidence les variations significatives suivantes :

- sous l'effet de l'effort :
  - une augmentation de l'hématocrite ;
- sous l'effet du traitement par l'acépromazine (chez le lot traité par rapport au lot témoin) :
  - une diminution : de l'hématocrite, du taux de la glucose 6 phosphate déshydrogénase,
  - une augmentation du taux de 2-3 diphosphoglycérate.

La discussion de ces résultats montre que les effets de l'acépromazine au niveau du métabolisme du globule rouge, apparaissent comme une conséquence de l'hypoxie induite par cette phénothiazine. De plus sous l'action de cette substance les mécanismes destinés à compenser l'anoxie tissulaire due à l'effort sont insuffisants pour exercer leurs effets.

### Introduction.

In equestrian sport, but particularly in dressage competitions and jumping events, the horse must be firmly under the rider's control, and to obtain calmer animals tranquilisers and neuroleptics are employed. This new form of doping, frequently repeated throughout the season, led us to study the effects of acetylpromazine on muscular activity in horse. Previous studies (Courtot *et al.*, 1975) showed that:

- the drug had eventually a toxic effect at the muscular level;
- acetylpromazine brought about depressed respiration.

These observations led us to pursue the work with the object of gaining a better understanding of the effects of acetylpromazine at the level:

- of the ECG (Mestries and Courtot, 1977);
- of cell metabolism.

The difficulties of experimentation on the horse limit the possibilities of working on muscle cells obtained by biopsy (Lindholm, 1974). As a consequence, we turned to the study of a more readily available cell: the erythrocyte. It was thus at the level of red cell metabolism that we looked for biochemical indicators to tissue oxygenation.

The change in the affinity of haemoglobin for oxygen in the Bohr effect, when the pH varies, is a composite effect of the three principal ligands of haemoglobin: oxygen, CO<sub>2</sub> and 2,3 diphosphoglycerate (2,3 DPG) [Poyart *et al.*, 1972].

Brewer (1974) underlined the importance of 2,3 DPG at the level of oxygen transport. Physiologically, 2,3 DPG is an allosteric modulator of the binding of oxygen to haemoglobin. 2,3 DPG binds strongly to deoxy-haemoglobin. The same author stressed that hypoxia associated with difference types of anaemia, with altitude, and with pulmonary disease, bring about an elevation in the level of red cell 2,3 DPG. It is generally accepted that the elevation in the level of 2,3 DPG in conditions leading to hypoxia is mainly responsible for the modification of

red cell oxygen affinity by increasing the half saturation pressure of haemoglobin (or P<sub>50</sub>) for the gas. This hypothesis has been confirmed in many species and in particular in the horse (Bunn, 1971).

Our previous work (Courtot *et al.*, 1975) showed that acetylpromazine brought about depressed respiration. This led us to make a special study of the effect of acetylpromazine on the levels of 2,3 DPG. Another important biochemical variable to consider is the intracellular concentration of reduced glutathione (GSH). Erythrocyte GSH plays an important role as a protector of protein sulphhydryl groups against oxidation, prevents the formation of methaemoglobin and maintains haemoglobin in a soluble functional state (Eaton and Brewer, 1974).

The activity of the pentose pathway is controlled by the proportion of oxidised glutathione: reduced glutathione (GSSG: GSH). Glutathione is necessary for the re-oxidation of the NADPH formed by this pathway. The metabolism of the red blood cell is purely anaerobic, there are no cytochromes, and a functional pentose pathway is essential for the survival of the red blood cell. The interruption (either by congenital defects or by drugs) of this metabolic pathway is frequently associated with haemolytic disease. The metabolite flow through the pathway is controlled by the first enzyme of the sequence: glucose-6-phosphate dehydrogenase (G6PD) (EC 111.49).

The object of the study reported here was to attempt to clarify the effect of acetylpromazine, administered at low levels within the context of doping, on the levels of erythrocyte 2,3 DPG, GSH and G6PD.

### Materials and Methods.

#### 1. COMPOSITION OF THE EXPERIMENTAL GROUPS

We used fourteen, five to thirteen year old horses of the "Selle Français" breed comprised of ten geldings and four mares. All be-

longed to the Société Hippique Nationale of Fontainebleau. Each horse was its own control. The first day the horse was exercised after an injection of physiological saline: "the placebo test". The following day the same amount of exercise was carried out after treatment with acetylpromazine: "the drug test". This order avoided residual effects of acetylpromazine and effects of climatic variations.

## 2. TREATMENT

Acetylpromazine was administered by deep intramuscular injection in the form of the maleate at a dose rate of 0.02 mg per kg live weight, one hour before beginning the test. At this dose rate, employed in doping, no clinical signs are visible.

## 3. TEST

The effort test took place in a riding school in order to be able to measure and standardize the work carried out. The distance covered was 1,680 metres, that is twelve circuits of 140 metres. The horse galloped at a constant speed of 340 metres per minute for five minutes.

## 4. EXPERIMENTAL SEQUENCE

The experiment was in three parts:

- a pre-experimental rest period of one hour;
- the effort-test lasting five minutes;
- a recovery phase limited to 45 mins.

Blood samples were taken from the jugular vein using heparinised Vacutainers during the experiment (Fig. 1).

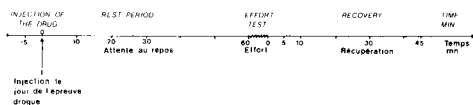


Fig. 1—The general layout of the experimental sequence. Blood samples were taken at the times indicated.

The horse was saddled and walked toward the schooling ring between the 45 minute and one hour times.

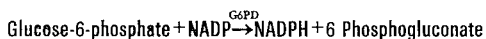
## 5. PARAMETERS MEASURED

(1) *Hematocrit*: The hematocrit was measured in blood collected in anticoagulant, in Wintrobe tubes, after centrifugation at 3,500 rpm for 10 mins.

(2) *Assay of reduced glutathione*: Reduced glutathione was measured by the method described by Roberts and Agar (1971). It is based on the formation of a stable derivative measurable at 420 nm. This derivative results from the reaction of 5-5 dithio-bis-2-nitrobenzoic acid (DTNB) with the sulphhydryl groups of reduced glutathione. The method was adapted for the Technicon auto-analyser and allows the direct measurement in whole blood.

The level of reduced glutathione is expressed as mg per 100 mls of red blood cells.

(3) *Measurement of glucose-6-phosphate dehydrogenase*: The assay was based on the method of Kornberg and Horecker (1955). The basis of the measurement is the following reaction:



The rate of appearance of NADPH, measured at 340 nm and 25°C, is proportional

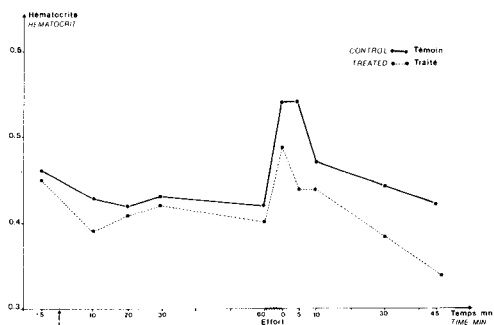


Fig. 2—Haematocrit variation as a function of time and treatment. I: injection of acetylpromazine. RBC: red blood cells.

to the activity of glucose-6-phosphate dehydrogenase in the haemolysate. The enzyme activity is expressed as millimoles of glucose-6-phosphate transformed per minute per ml of whole blood.

(4) *Measurement of 2,3 diphosphoglycerate:* 2,3 diphosphoglycerate was assayed by an enzymatic technique (Nygard and Rorth, 1969). The measurement was carried out on haemolysates obtained by diluting whole blood with 100 volumes of water oxygenated by agitation in air at ambient temperature.

The results are expressed in millimoles of 2,3 DPG per litre of red blood cells.

6. STATISTICAL ANALYSIS

The statistical analysis of the results was carried out by a standard analysis of variance of two factors: treatment and time. This analysis of variance was carried out on the mean of each group: control (horses the day of the "placebo test") and treated (same horses the day of the "drug test").

Results.

1. HAEMATOCRIT (Fig. 2).

The haematocrit value increases sharply after the effort. The return to normal is

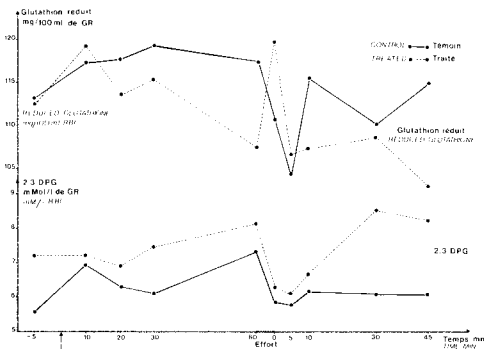


Fig 3—Variation in the level of reduced glutathione and of 2,3 diphosphoglycerate (2,3 DPG) as a function of time and treatment. I: injection of acetylpromazine. RBC: red blood cells.

very rapid ( $F_0=5.27$  significant at a level of 2.5 per cent).

For the treated group the haematocrit was inferior to that of the control group ( $F_0=19.64$  significant at a level of 1 per cent). The difference between the two groups increase to a maximum at the end of the recovery phase (45 minutes after the test effort).

2. REDUCED GLUTATHIONE (Fig. 3).

The level of reduced glutathione shows no significant variation either as a function of time ( $F_0=1.66$ ) or of treatment ( $F_0=1.52$ ).

3. 2,3 DIPHOSPHOGLYCERATE (Fig. 3).

The level of 2,3 DPG in the treated animals was always higher than those of the controls ( $F_0=17.6$  significant to a limit of 1 per cent). This difference was clear during the recovery phase where the level of 2,3 DPG fell after the test and then increased in the 30 minutes which followed the test effort. This variation as a function of time was not visible in the controls and the variation was thus not significant ( $F_0=2.28$ ).

4. GLUCOSE-6-PHOSPHATE DEHYDROGENASE (Fig. 4).

Variations of G6PD as a function of time were not significant ( $F_0=0.76$ ). The acetyl-

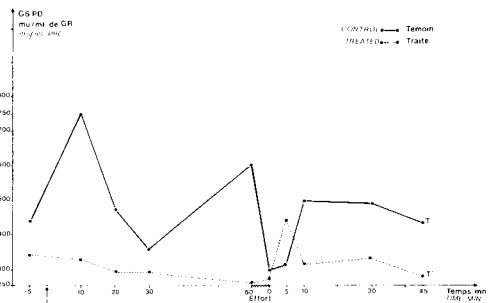


Fig 4—Variation in the activity of glucose-6-phosphate dehydrogenase as a function of time and of treatment. I: injection of acetylpromazine. RBC: red blood cells.

promazine treatment led to a lower and above all less stable level of G6PD ( $F_{1,9} = 19.64$  significant to a limit of 1 per cent). An increase appeared consistently five minutes after the test effort in the treated animals.

### Discussion.

The experimental protocol employed allows for the difficulties inherent in the usage of the riding horse as an experimental animal. The variability between individuals and the low numbers of experimental animals at our disposal led us to devise an experimental plan whereby each animal was its own control. Thus statistical analysis based on the use of the difference before and after treatment is certainly the most appropriate. But the missing data gravely limit this approach.

For this reason we carried out an analysis of variance of the average values calculated on the day of the "placebo test" (controls) and the day of the "drug test" (treated) respectively. The analysis of our results allowed us to show up the following significant variations:

- under the effect of the test effort:
  - an increase in the haematocrit;
- under the effect of acetylpromazine treatment (in the treated group by comparison with the control group):
  - a decrease: of the haematocrit, the level of glucose-6-phosphate dehydrogenase and a tendency towards a decrease (not significant) in the level of reduced glutathione,
  - an increase in the level of 2,3 diphosphoglycerate.

Our discussion is based on the examination of these results.

### HAEMATOCRIT

In the horse at rest, 33 per cent of the red cells are stored in the spleen. With exercise or fright and by adrenalin release, splenic contraction brings about an increase in the circulatory red blood cell numbers (Torten, 1964). This release of red blood cells into the blood circulation increases the oxygen transporting capacity (Milne, 1976).

In the horse at rest, the packed red blood

cell volume oscillates between 36 and 43 per cent. Fifteen minutes after intense muscular effort it attains 50 to 56 per cent (Hamm, 1972). Murakami and Takagi (1974) show that the haematocrit increases progressively up to the middle or the end of exercise and then returns progressively to the initial level within one hour. In the present study we observed the same pattern.

Nevertheless, the injection of acetylpromazine brought about lower haematocrit levels. This observation can be explained as being brought about by a decrease in the anxiety of the animals.

Haematocrit decreases under the influence of a neuroleptic have been described in other species with respect to:

- propionylpromazine in cattle and in the horse (De Moor and Hende, 1968);
- chlorpromazine in the dog (Collette and Meriwether, 1965); (Turner and Hodgetts, 1960), in cattle (Gartner and Ryley); (Beattie, 1975), in sheep (Turner and Hodgetts, 1960). The same phenomenon was described by Lees and Serrano (1976) with respect to a butyrophenone:azaperone in the horse.

All these authors agree that the haematocrit decrease is due to the adrenalytic effect of the phenothiazines.

### GLUCOSE-6-PHOSPHATE DEHYDROGENASE

As far as we are aware the effect of effort on G6PD has not been described in literature. In our study we have observed no significant effect of effort on the activity of this enzyme.

How can the decrease in activity of G6PD after acetylpromazine treatment be explained? This enzyme depends on the intracellular concentrations of glutathione (Jacob and Jandl, 1964), ATP and magnesium (Avigad, 1966). Eaton and Brewer (1974) summarized the factors influencing G6PD activity and these are shown below:

Factors whose decrease in concentration inhibits G6PD: NADP, GSSG, Ascorbate, Oxygen, Glucose-6-phosphate, Magnesium.

Factors whose increase in concentration inhibits G6PD: Catalase activity, ATP, 2,3 DPG.

From amongst these multiple factors we

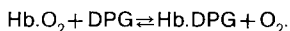
know the effects of acetylpromazine on oxygen and 2,3 DPG.

Acetylpromazine brings about a depression of respiration (Courtot *et al.*, 1975; Mestries and Courtot, 1977) and a decrease in the haematocrit. Both these phenomena lead to a decrease in the erythrocyte oxygen levels. The level of 2,3 DPG is increased by acetylpromazine. But these changes, both which tend to cause a decrease in the activity of G6PD, are insufficient in themselves to explain the decrease since the enzyme is dependant on multiple factors.

### 2,3 DIPHOSPHOGLYCERATE

The level of 2,3 DPG found in our experiment (6 mMoles/litre of red blood cells) is close to the values reported by Bunn (1973) in the horse:  $6.40 \pm 0.86$  mMoles/litre red blood cells. These values are similar to those reported for man: 5 mMoles/litre erythrocytes (Vallet and Callis, 1973).

Lenfant *et al.* (1971) noted that in man hypoxia due to high altitude stimulated glycolysis and as a consequence the synthesis of 2,3 DPG so that erythrocyte levels rose, facilitating the dissociation of oxyhaemoglobin and thus tissue oxygenation, following the chemical equilibrium represented below.



But hypoxia only provokes this increase when it is associated with physical effort. This has been shown experimentally in the rabbit (Vallet and Callis, 1973).

In our study acetylpromazine brings about a decrease in the respiration rate, from which a hypoxia associated with effort could explain the increase in 2,3 DPG which we observe, and this leads to a compensatory increase in the  $P_{50}$  with respect to oxygen.

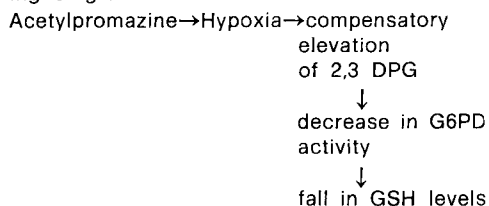
### REDUCED GLUTATHIONE

In the pentose pathway G6PD converts glucose-6-phosphate to 6-phosphogluconolactone. During the reaction NADP is re-

duced to NADPH; this cofactor is used to maintain glutathione in its reduced form (GSH).

In our study the decrease in GSH levels is not significant but numerous factors have a role in its regulation. Nevertheless, there is a tendency for a decrease during the recuperation phase following the test effort after acetylpromazine treatment.

*To summarize:* The discussion of our results shows that the effects of acetylpromazine at the level of the erythrocyte metabolism appear to be a consequence of the hypoxia induced by this phenothiazine derivative. Schematically we can explain the action of acetylpromazine by putting forward the hypothesis shown in the following diagram:



### Conclusion.

After previous work has revealed that acetylpromazine brought about a decrease in respiration rate, we looked for biochemical effects of this drug at the level of the red cells. The results obtained demonstrated that under the action of this substance the mechanisms designed to compensate for the tissue anoxia following effort have reduced effectiveness.

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### Summary

The repeated use of tranquillisers and neuroleptics during equestrian events led to the

study of the effects of acetylpromazine on muscular activity in the riding horse. Previous work (Courtot *et al.*, 1975) concludes that:

- there was eventually a toxic effect of the drug at the muscular level manifested by an increase in the level of creatine phosphokinase in serum;
- respiratory depression appeared.

These observations lead us to continue the work and to study the effect of acetylpromazine at the level:

- of cell metabolism;
- of the ECG.

The object of the present study was to examine the effect of acetylpromazine at the level of the erythrocyte and in particular:

- the cell's level of 2,3 diphosphoglycerate (2,3 DPG);
- the activity of red cell glucose-6-phosphate dehydrogenase (G6PD);
- the level of reduced glutathione (GSH).

The evolution of these variables was studied before and after a standardized test effort. Acetylpromazine was injected at a low dose rate (0.02 mg/kg) one hour before the beginning of the test effort.

Analysis of the results revealed the following significant changes:

- as a result of the test effort: an increased haematocrit;
- as a result of treatment with acetylpromazine (in the treated compared with controls):
  - a decrease: of the haematocrit and the level glucose-6-phosphate dehydrogenase,
  - an increase in the level of 2,3 diphosphoglycerate.

Discussion of these results show that the effects of acetylpromazine at the level of erythrocyte metabolism appears as a consequence of hypoxia induced by this phenothiazine derivative. It seems that under the action of the drug the mechanisms designed to compensate for tissue anoxia due to effort are insufficient to produce their effects.

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