primary immune and anamnestic responses in the mouse infected with *T. spiralis*.

Materials and methods. Swiss-Webster male albino mice were used throughout. Recovery of larvae from stock mice and infection procedures were according to HARLEY and GALLICCHIO[3]. 20 mice were infected by gavage with 350 *T. spiralis* larvae. After 30 days postinfection, 10 of these mice were desiginated Group I (immunized controls) and 10 Group II (immunized + azathioprine).

Stock mice which had not been infected were divided into Groups III (control, nonimmunized) and IV (control + azathioprine), with 10 mice per group. Each mouse received, by gavage, 350 *T. spiralis* larvae. Groups II and IV were simultaneously treated orally with 3 mg/day of azathioprine (Imuran®, Wellcome Research Laboratories) beginning 2 days preinfection and continuing daily through day 20 postinfection.

All mice were killed 30 days postinfection and the number of muscle larvae determined according to HARLEY and GALLICCHIO[3]. Student's *t*-test was used to determine the statistical significance of the observed differences in numbers of muscle larvae recovered from experimental and control groups. A probability greater than 0.05 was not considered significant.

Results and discussion. The effect of azathioprine on numbers of *T. spiralis* muscle larvae in immunized vs nonimmunized mice is shown in the Table. Statistically, there was no significant ($P > 0.05$) difference in the number of muscle larvae in immunized, nonsuppressed mice (Group I) as compared to nonimmunized, suppressed mice (Group IV). All other differences were significant ($P < 0.05$).

Effect of azathioprine on numbers of *Trichinella spiralis* muscle larvae in immunized vs nonimmunized mice

Group	Mean ± S.D.
Immunized mice + 350 *T. spiralis* larvae (I)	715.832 ± 199.490
Immunized mice + 350 *T. spiralis* larvae + azathioprine (II)	327.191 ± 97.813
Normal mice + 350 *T. spiralis* larvae (III)	499.703 ± 177.797
Normal mice + 350 *T. spiralis* larvae + azathioprine (IV)	945.971 ± 266.164

Thus, azathioprine suppressed the primary immune response of mice infected with *T. spiralis*; however, the anamnestic response was not suppressed. This study gives further evidence that immunity in mice infected with *T. spiralis* is primarily due to delayed (cellular) hypersensitivity. This latter response leads to intestinal inflammation and subsequent expulsion of adult worms. Since the number of muscle larvae was significantly reduced ($P < 0.001$) in immunized, suppressed mice as compared with the immunized nonsuppressed mice, the anamnestic response was not affected.

This again gives further support to the accumulation of evidence that the inflammatory change in the intestine is the final effector mechanism of the delayed hypersensitivity reaction. That this delayed hypersensitivity exists, supports the hypothesis that 'the immunity of mice against the adult worms of T. *spiralis* has a cell-mediated basis'[1]. Finally, this described host-parasite model system can be a useful adjunct to evaluate the immunosuppressive action of other experimental drugs that are designed to inhibit the hypersensitivity phenomena and/or antibody formation[2].

Zusammenfassung. Die Wirkung von Azathioprin auf die Immunantwort von mit *Trichinella spiralis* infizierten Mäusen ist auf Grund der Anzahl in der Muskulatur aufgefundenen Larven beurteilt worden. Nach einer Erstinfektion zeigen behandelte Tiere gegenüber Kontrollen eine unterdrückte Immunantwort. Eine immunsuppressive Wirkung von Azathioprin bleibt bei einer sekundären Infektion hingegen aus.

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[3] J. P. HARLEY and V. GALLICCHIO, Expl. Parasit. *30*, 11 (1971).
[4] To whom reprint requests should be sent.

Control of Anticoagulation in Rats

Coumarins are widely used as rat-killers by inducing severe hypocoagulability which results in haemorrhaging, but socalled therapeutic anticoagulation in the rat, as one of the most frequently used laboratory animals, has scarcely been studied. For the laboratory control of the degree of anticoagulation in rats, thromboplastins[1-4] were used, which were not well-defined as to their sensitivity for rat coagulation factors.

Experience in humans has been that calibration of thromboplastins is rather difficult[5]. Sensitivity for clotting factor levels has to be checked as well as the inhibitory influence of inactive clotting factors, called PIVKAs (Proteins in vitamin K-absence; Hemker et al.[6]). We decided to evaluate a rapid and well-standardized assay method for its sensitivity for the mean clotting factor level of the prothrombin complex[7] and the possible influence of PIVKA on the test result in rats under anticoagulation.

Methodology. A) For the construction of a reference curve for blood samples assayed in the Normotest reagent (Nyco, Oslo, Norway) 12 healthy male Wistar rats (aged 4 months, body weight 325–375 g) from an inbred colony[8] fed on a standard diet and with factor II, VII and X levels in the normal range (80–120% assayed against

[1] B. B. COLDWELL and Z. ZAWIDSKA, Blood *32*, 945 (1968).
[2] Z. Z. ZAWIDZKA, B. B. COLDWELL and H. C. GRICE, Experientia *28*, 1482 (1972).
[3] TH. J. MCINTOSH, W. R. WILSON, L. WATERS and J. R. FOUTS, Eur. J. Pharmac. *14*, 176 (1971).
[4] R. NIEDNER, M. KAYSER, N. REUTER, FR. MEYER and W. PERKOW, Arzneimittel-Forsch. *23*, 102 (1973).
[5] E. A. LOELIGER, Thromb. Diath. Haemorrh. *28*, 109 (1972).
[6] H. C. HEMKER, J. J. VELTKAMP, A. HENSEN and E. A. LOELIGER, Nature, Lond. *200*, 589 (1963).
[7] J. J. VELTKAMP, in *Human Blood Coagulation* (University Press, Leiden 1969), p. 381.
[8] H. G. M. GEERTZEN, F. J. G. VAN DER OUDERAA and A. A. H. KASSENAAR, Acta endocr., Copenh. *72*, 197 (1973).

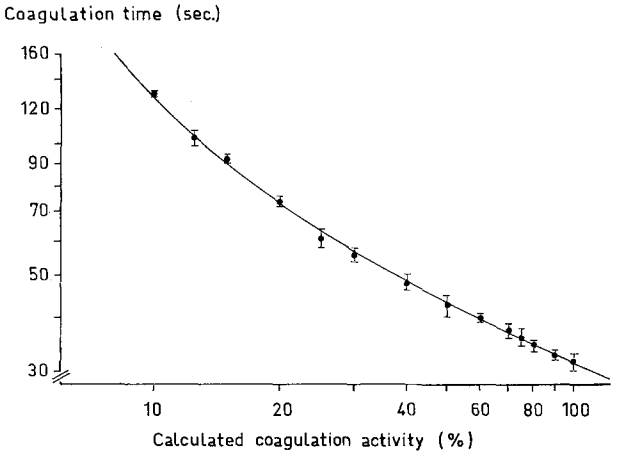

Fig. 1. Saline dilution curve of rat blood asasyed in the Normotest reagent.

pooled plasma derived from 30 normal rats) were punctured under ether anesthesia in the aorta and 4.9 ml blood was collected in polystyrol crystal tubes containing 0.1 ml 20% trisodium citrate dihydrate solution. From every blood sample 0.020 ml was blown into 0.25 ml Normotest reagent and the clotting time was read. The same procedure was carried out with 75, 50, 25 and 12.5% saline dilutions of the individual samples. Subsequently all blood samples were mixed and the Normotest was performed for 90, 80, 70, 60, 50, 40, 30, 20, 15, and 10% dilutions of this mixture. All assays were duplicated.

B) Two types of experiments were done for the construction of other curves: 1. 6 groups of 7 rats, kept under the same conditions as mentioned above, were anticoagulated with Warfarin (Coumadin-sodium, Endo-lab, New York, USA) injected i.p., in a dose of 150 µg/100 g body wt. This dose must be considered as totally blocking, since doses of 100 µg/100 g body wt. and more gave identical disappearance rates of the clotting factors[9]. At 3, 6, 9, 14 and 24 h blood was sampled under ether anesthesia from the tail by severing one vertebra. Firstly a hematocrit capillary (Gelman-Hawskley, Lancing, England) was filled. Secondly a 20 µl pipette (Dade, Miami, USA) was filled with blood and a Normotest assay was carried out immediately. Thirdly 0.15 ml blood was sampled by the method of PYÖRÄLLÄ[9] with the dilution fluids of DYGVE and LUND[10] to assay the coagulation factors II, VII and X. These citrate dilution fluids (final conc. 7.9 mM) were buffered with 9.9 g/l Hepes (Calbiochem, Los Angeles, USA)[11] and also Trasylol 550 IU/l (Bayer, Leverkusen, Germany) was added to inhibit contact activation[12]. The factor II, VII and X assays were carried out in a one-stage method, using artificial substrate plasma's[13,14] and home-made rat-brain thromboplastin according to the method described by OWREN and AAS[15] for human brain thromboplastin. The normal reference plasma for these assays was obtained by aortic puncture under ether anesthesia of 30 normal rats, using PYÖRÄLLÄ's method[9].

2. Three groups of 7 rats under the same conditions got 25, 25 and 15 µg Warfarin/100 g body wt. on the first 3 days respectively. At the fourth day blood was sampled as in the previous experiment and they were continued on Warfarin in an individual dose (mostly between 12 and 15 µg/100 g body wt. per day) for 3 weeks. Every 3 days a blood sample was taken and the dosage scheme adapted. When hematocrit values dropped more than 5%, the coagulation parameters were rejected. After blood sampling the tail was cauterized by an electrocoagulator Engel (type MK310).

[9] K. PYÖRÄLLÄ, Annls Med. exp. Biol. Fenn. *43*, suppl. 3. (1965).
[10] H. DYGVE and E. LUND, Acta med. scand. *150*, 207 (1954).
[11] S. ZUCKER, M. H. CATHEY and B. WEST, J. clin. Path. *53*, 924 (1970).
[12] C. R. M. PRENTICE, G. P. MCNICOL and A. S. DOUGLAS, Thromb. Diath. Haemorrh. *24*, 265 (1970).
[13] F. KOLLER, E. A. LOELIGER, F. DUCKERT, Acta haemat. *6*, 1 (1951).
[14] H. C. HEMKER, A. C. W. SWART and A. J. M. ALINK, Thromb. Diath. Haemorrh. *27*, 205 (1972).
[15] P. A. OWREN and K. AAS, Scand. J. clin. Lab. Invest. *3*, 201 (1951).

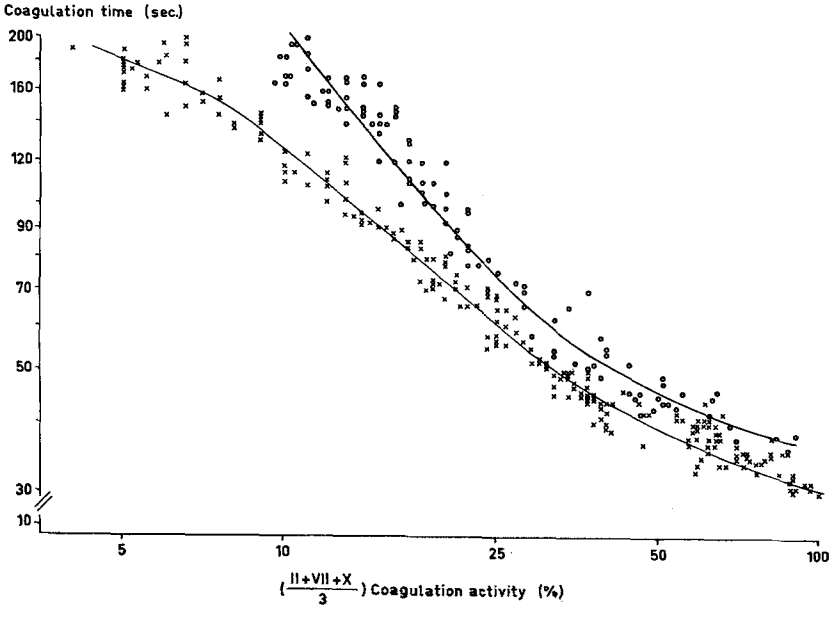

Fig. 2. Normotest results obtained with blood samples from warfarin-anticoagulated rats plotted against the mean activity of the clotting factors II, VII, and X assayed separately with a specific method. The lower curve (X) is based on the results during the initial stage of anticoagulation. The upper curve (○) represents the results during longterm treatment. Each symbol represents 1 blood sample.

Results. Test results in the Normotest assay for serially diluted individual and pooled blood samples, obtained by aortic puncture in normal rats, were used to construct a reference curve by plotting the clotting time against calculated coagulation factor activity (Figure 1). The results of the individual and the pooled samples fit the same hyperbolic curve.

The Normotest coagulation times in blood samples taken from the tail of rats on anticoagulant therapy were plotted against the mean plasma level of factors II, VII, and X assayed in a simultaneously collected sample. The reference curve for the initial stage of anticoagulation, using a warfarin dose completely blocking[9] the clotting factor synthesis, can be seen in Figure 2; it is identical in shape with the saline curve until very low levels of coagulation factors are reached and relatively too short Normotest times are found.

The data obtained in long-term anticoagulation, from 4 to 21 days, yield a curve that deviates from the other curves, especially in the area below 20% coagulation factor activity, in the sense that Normotest times are relatively too long (Figure 2).

During the long-term treatment, from the 12th day on, succesively 14 of the 21 rats showed a drop in hematocrit and died from bleeding (confirmed on autopsy).

Discussion. A reagent for the control of anticoagulant therapy should be sensitive for the depression of clotting factors of the species treated. For the control in rats, we found human brain and ox-brain thromboplastin (Thrombotest) unsuitable because of the flatness of the standard reference curve. Rat-brain thromboplastin, laborious to produce, and rabbit-brain thromboplastin (Normotest) appeared to be sufficiently sensitive. The only modification in the Normotest assay procedure was the use of 20 µl blood as compared to 10 µl used for human blood. With 10 µl of rat blood in the lower clotting factor activity levels were too long and irreproducible clotting times were found.

The saline dilution curve assayed in Normotest, a reagent not sensitive to changes in factor V and fibrinogen, should be identical to a curve constructed with test-results in anticoagulated rats, which is indeed the case for the curve constructed with data from the initial phase of anticoagulation. Below the values of 10% coagulation factor activity, relatively too short Normotest times are found, which can be explained by the fact that these rats were bled 5 times within 24 h which caused a drop in hemotocrit value, amounting to about 4%, giving relatively more plasma in the samples[16].

An essential deviation from both the saline curve and the curve based on the initial stage of anticoagulation is observed for the curve based on long-term anticoagulant samples, especially below the 20% coagulation factor level, which we believe to be caused by inhibition of the Normotest assay by PIVKAs.

It could be argued that in long-term anticoagulation all 3 factors show equally depressed activity, whereas in the initial stage factor VII (fastest turnover rate[9]) activity will decrease most rapidly. Even when we excluded factor VII results in the calculations, we found the same discrepancy between the 2 curves.

It might be that PIVKAs in the rat appear in a later stage of anticoagulation as compared to humans[17], because also SUTTIE[18], using immunochemical methods, could not demonstrate abnormal prothrombin (PIVKA-II) during the initial stage of anticoagulation in rat blood.

For practical purposes, e.g. the study of interactions between other drugs and oral anticoagulants, rats should not be anticoagulated below 15% activity of the coagulation factors of the prothrombin complex, which corresponds to 145 sec Normotest, because of the high bleeding incidence. Furthermore it should be realized that Normotest times in the initial stage of anticoagulation differ so much in significance from those during long-term treatment that the 2 different curves have to be used[19].

Résumé. La méthode d'analyse à l'aide du réactif Normotest du contrôle en laboratoire de l'anticoagulation par les dérivés des cumarines montre chez les rats une sensibilité propre pour la diminution de l'activité des facteurs de coagulation et aussi pour l'action inhibitrice des PIVKA, lesquels se manifestent dans le sang avec un certain délai. Un traitement de longue durée à moins du 15% en moyenne de l'activité des facteurs de coagulation est déconseillé à cause des très grands risques d'hémorragie.

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[16] N. TRYDING, R. BERG, J. E. NILSSON, S. E. NILSSON, O. K. STRANDLI and G. TULVESSON, Farmakoterapi *25*, 27 (1969).
[17] M. BROZOVIC and L. GURD, Lancet *2*, 427 (1971).
[18] J. W. SUTTIE, Science *179*, 192 (1973).
[19] The authors wish to thank Mr. W. ZUIDERVAART for his invaluable assistance in the animal handling and Mrs. N. GOEDHART-DE GROOT and Mr. N. H. VAN TILBURG for their assistance in the Normotest assays.

Effect of Cytosine Arabinoside on Thyroid Autoimmunity in Guinea-Pigs

Cytosine arabinoside (Ara-C), a pyrimidine nucleoside analog, selectively interfers with DNA synthesis at the S-phase of the cell cycle[1]. The primary effect of this antimetabolite in all mammalian species is exerted on the bone marrow and other rapidly dividing cell populations[2]. Regarding the immune response, Ara-C has been found to have a marked effect on the rapidly dividing lymphoid cells during the log phase of humoral antibody production[3]. In thyroid antoimmune systems, antimetabolites have been shown to be uniquely qualified as agents to probe the various phase of the autiommune response[4]. Ara-C has been successfully employed as an immunosuppressant in allergic encephalomyelitis but is untested concerning autoimmune thyroiditis[5]. In view of these findings, the present study was designed to investigate the effects of Ara-C on thyroid autoimmune induction by i.p. ad-

[1] E. FREI, H.N. BICKERS, J.S. HEWLETT, M. LANE, W.V. LEARY and R.W. TALLEY, Can Resour. Bull. *29*, 1325 (1969).
[2] C.G. SMITH, in *The Control of Growth Processes by Chemical Agents* (Pergamon Press, New York 1968), p. 33.
[3] J.E. HARRIS and E.M. HERSH, Can. Resour. Bull. *28*, 2432 (1968).
[4] P. MIESCHER, F. GORSTEIN, B. BENACERRAF and P.G.H. GELL, Proc. Soc. exp. Biol. Med. *107*, 12 (1961).
[5] E. MIHICH, in *Principles of Immunology* (Eds. N. ROSE, F. MILGROM and C. VAN OSS; MacMillan Publ. Co., Inc., New York 1973), p. 364.