The presence of androsterone in the third spot was confirmed by gas-liquid chromatography and by the Zimmermann reaction. These steroids were present in the tissue in the following concentrations, calculated from the area under the curve in gas-liquid chromatography: progesterone 34.3 μg/kg, 20β-hydroxypregn-4-en-3-one 4.5 μg/kg, androstenedione 53.7 μg/kg, pregnenolone 123.4 μg/kg, dehydroepiandrosterone 454 μg/kg, androsterone 99 μg/kg.

The fourth spot had a very strong UV-absorption maximum at 240 nm in absolute ethanol, but no testosterone could be detected in gas-liquid chromatography. One spot was detected in the conjugated fraction with Rf corresponding to testosterone. The UV-spectrum in ethanol showed an absorption maximum at 240 nm; the oxidation product showed the same Rf of androstenedione on thin-layer chromatography; finally the gas-liquid chromatographic analysis confirmed the presence of testosterone at a concentration of 91 μg/kg.

Phenolic fraction. Both free and conjugated fractions were examined for the presence of oestradiol-17β, oestrone and oestriol. The thin-layer chromatography system used was ethanol-benzene 10:90. The spots corresponding to the 3 oestrogens were eluted, and the UV-spectra in NaOH 0.5 *N* in 80% ethanol were taken. Part of the extract was used for gas-liquid chromatography. By these methods it was possible to detect oestradiol-17β in the free fraction in a concentration of 127 μg/kg and oestrone in both the free and the conjugated fractions in total concentration of 326 μg/kg.

The present investigation has demonstrated that androgenic as well as oestrogenic substances are present in lizard testicular tissue. Testosterone has been shown to be present in the conjugated fraction only. Of particular interest is the ratio progesterone/androstenedione/testosterone, which approaches that of mammals. In fact, in the other lower vertebrates so far examined, androgen precursors are present in a concentration higher than that of testosterone; for instance in *Scyliorhinus stellaris* testes the ratio of these steroids is 100:70:50 (μg/kg)[4], while in *Lacerta sicula* testes it is 34.3:53.7:91 (μg/kg)[6].

Riassunto. Gli autori hanno analizzato gli steroidi presenti nel tessuto testicolare di *Lacerta sicula*. Sono stati identificati i seguenti steroidi: progesterone, androstenedione, testosterone, androsterone, deidroepiandrosterone, pregnenolone, 20β-idrossipregn-4-ene-3-one, estradiolo-17β, estrone. Viene discusso il rapporto progesterone androstenedione testosterone, paragonandolo a quello trovato in altri vertebrati inferiori.

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Institute of Zoology and Comparative Anatomy, University of Camerino, and Zoological Station, Naples (Italy), July 19, 1966.

[6] This investigation was supported by a research grant HD 01477-06 from the National Institute of Child Health and Human Development, Public Health Service, USA, and by the Consiglio Nazionale delle Ricerche of Italy.

PRO EXPERIMENTIS

An Apparatus for Direct X-Ray Cinematography Exemplified by Analysis of Some Respiratory Movements in *Gasterosteus aculeatus*

Measurement of movement between elements within intact organisms poses an important problem for functional anatomists. It is always preferable to make such observations in undisturbed animals; often this is absolutely essential. One of the many techniques successfully applied is X-ray cinematography, utilizing an image intensifier[1-3]. However, this technique has limitations in terms of the distinctness of image detail obtainable by present methods.

Direct application of X-rays provides markedly increased resolution, but it can hardly be used in human radiology because of the high dosages required[3]. Since the animals used for these experiments have much thinner bones and much greater tolerance, the dosage required may be permissible. The apparatus, which will be described below, allows one to obtain a rapid sequence of direct X-ray exposures of a small moving object. The results obtained are demonstrated by the analysis of the respiratory movement of 2 cranial elements of the 3-spined stickleback, *Gasterosteus aculeatus*.

X-ray motion pictures of the movements of bony elements may be obtained by the use of two comparable techniques. The indirect technique, applied increasingly in medical diagnostics, utilizes an image intensifier. X-rays impact on a screen covered by a glass tube. Behind the screen is placed a photocathode which releases electrons in an amount depending on the intensity of the X-rays. The electrons are concentrated on and accelerated to a second small screen, which transduces the image to the visible region. It is then observed by means of an optical system, or a television camera, or filmed with a cine-camera. The intensification of brightness of the image on the second screen permits the use of normal blue sensitive films with low kilovoltage and low tube current, thus small X-ray dosage.

The limitation of this technique is due to its indirectness. In spite of their fine grain material, the 2 screens cause an unsharp image. Moreover, an important additional unsharpness is caused by different kinds of 'noise'. First there is considerable variation in the X-ray quanta falling on the first screen, secondly there is directional variation during the emission of electrons. This type of unsharpness can include indistinctiveness and loss of

[1] J. NAUTA, Res. Film *1*, 3 (1953).
[2] G. J. VAN DER PLAATS, *Medical X-Ray Technique* (1959).
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contrast[2,3]. For medical purposes the benefits of the image intensifier greatly exceed the limitations. For observing the details of the movement of bony parts in small animals this technique is insufficient.

In the direct technique the films are exposed to the X-rays directly. In medicine the well-known uninterrupted film strip is sometimes used. According to VAN DER PLAATS[2] 'this method requires complicated equipment and is almost prohibitively expensive'. Apart from these drawbacks it has further limitations in the special sensitive films and the high dosage of X-rays that must be used. For the last reason it is almost impossible to apply this technique in medicine.

However, in experimental circumstances with animals possessing thin bones, the required dosages are within the physiologically tolerable range. In 1962 FOXON[4] constructed a simple effective apparatus for direct X-ray serial exposures. A strip of film was moved in a lead tube in which an exposure hole was made. By moving the film a shutter was moved synchronously over the hole. For a number of reasons, stated below, we found this apparatus unsuitable for our research.

Using the direct technique, we have now developed another apparatus which enables us to make a sufficient number of pictures on X-ray film for a relatively fast movement.

The main principle of this apparatus is the independence of the exposure and the transportation (Figures 1 and 2) of a great number of films in a short time. In this it contrasts to the strip film method[4]. The principal components of the apparatus are the transportation system of the unexposed films, the exposure system, and the transportation system of the exposed films. These 3 components are in principium independent. In particular, the 2 transportation systems do not influence each other mechanically. This construction has the advantage that the speed of movement for the first transportation system can be low and is reduced by small forces, simplifying attainment of a motionless position of the films during exposure and permitting the use of relatively long exposure times. After exposure the films can be removed at a much higher speed.

These principles are realized by exposing the uppermost film of a stack of package sheet films, and removing it at right angles to the direction of feed after exposure and then exposing the next uppermost film. The column of films is moved upward in a rectangular perspex tube (1) by a piston (2) under air pressure. A simple electrical valve with a switch controls the pressure. Low pressure is sufficient to ensure a smooth movement of the column. In the exposure window (3) the films are pressed against 2 brass bars (10) supporting the films only on the top side. Lateral support would counteract a centrifugal force during removal. This force would cause a vibration leading to unsharpness. Consequently lateral support was omitted.

As loose single films are used, the transportation of a rather inert mass did not pose a problem. No influence of the transportation mechanism on the film under exposure was observable, so that no unsharpness by movement occurred.

4 brass strips (5) arranged on the underside of a rotating massive brass wheel (4) serve to propel the films from the column. 4 holes (6) in the wheel represent the shutter mechanism. The distance between hole and strip is equal to film length, so that the film is covered by the radioopaque wheel before transportation. At least the same distance is left between the strip and the next hole. The films slide through a special groove (7) and are collected in a bag. The mass of the wheel ensures a constant velocity which can be determined by an adjustable transmission system (8). The wheel is allowed to rotate during preparation time. Increase of air pressure moves the column of films upward into position and they are exposed. Thus the investigator can choose the right moment of exposure, e.g. when the animal happens to show the movement wanted.

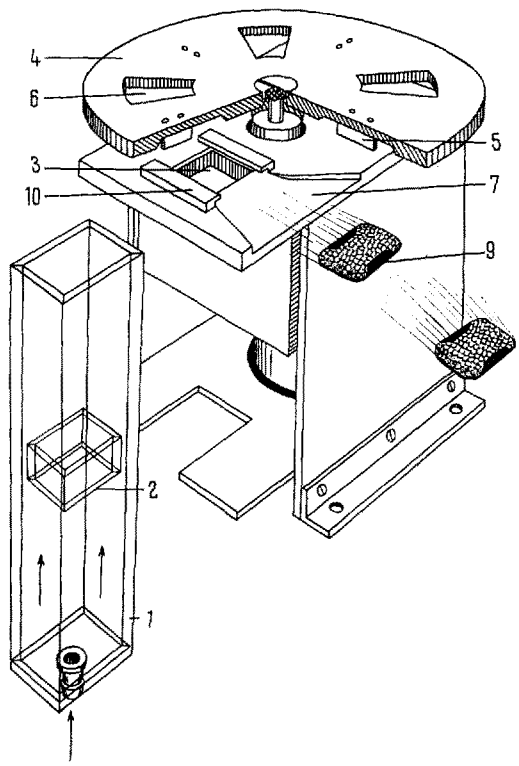

Fig. 1. Diagrammatic drawing of the apparatus.

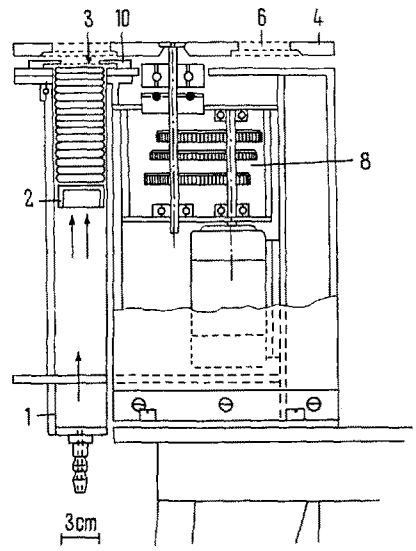

Fig. 2. Longitudinal section of the apparatus.

[4] G. E. H. FOXON, Br. J. Radiol. *21*, N.S. (1948).

Each piece of Kodak dental X-ray film is 3 · 4 cm in size and is covered at the bottom side by a thin sheet of lead. Film and lead are packed in a pertinax envelope (9). Pertinax is a material equivalent to NEMA, XXXP, a purified laminate phenolic resin impregnated with x-cellulose. The packing has the advantage of a better sliding of the films and a better resistance against the impact of the vertical strips (5). Care was taken that sharp edges of the strips could not damage the films. Films are numbered beforehand with punched numbers. After development in Kodak DX 80, exposure data 80 kV, 100 mA, focus film distance 25 cm, focus 1.2 of Philips Rotalix tube, Standard DLX, the order may be established again.

The animal is placed in a pertinax tube on an independent pertinax table close to the wheel, thus preventing geometric unsharpness. Films were exposed about $^1/_{70}$ sec at 42 frames/sec at a rotation speed of 10.5 c/sec. The rather quick respiratory movement of a stickleback could be filmed with satisfactory results.

The size of the frame is rather small, although it exceeds the effective surface of a 35 mm normal movie-film.

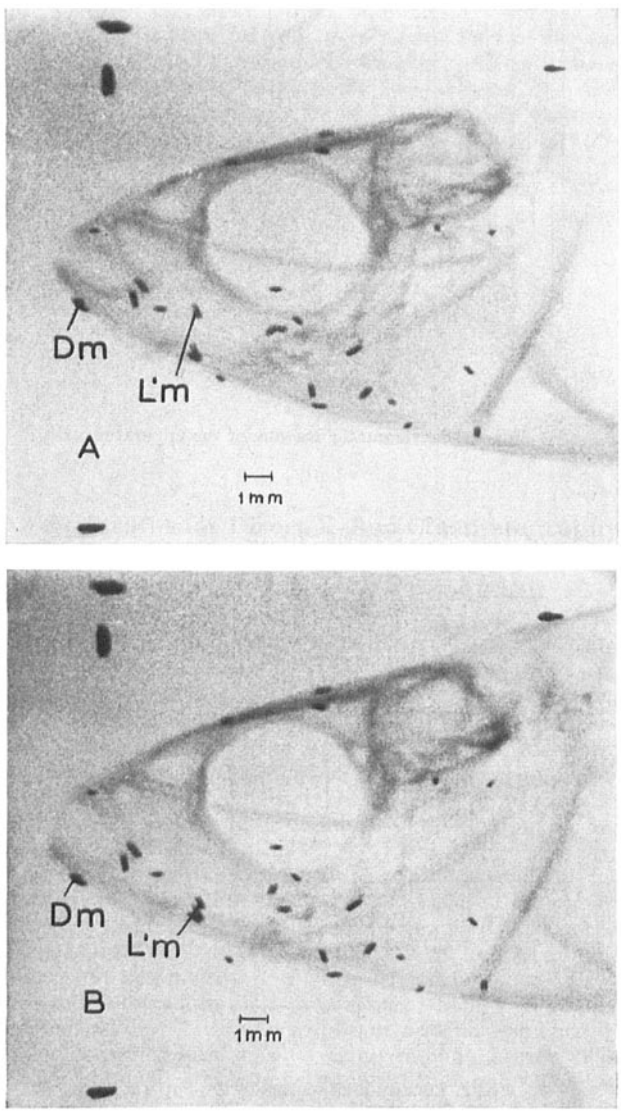

Fig. 3. Radiographs of the head of *Gasterosteus aculeatus* with open (B) and closed (A) mouth. Dm and L'm refer to the measured points (see Figure 4 and text).

However, preference was given to an easily obtainable film, which could be packed conveniently. No doubt, improvements can be obtained by using other films of greater size and finer grain. It is hoped to improve this apparatus further, especially from the viewpoint of dosage, exposure time and duration of filming, by utilizing a cine-pulse X-ray source.

As an example of the results obtained, Figure 3 shows 2 frames taken from a series of a respiratory cycle of *G. aculeatus*. The animal was clamped behind the pectoral fins in a small pertinax tube, thus fixing it at a determined spot. Circumstances, e.g. exposure time, temperature and oxygen supply, were kept as constant as possible during the lateral shooting. On the pictures skeletal elements are distinct. Unsharpness is caused by grain size of the film, by absorption owing to the surrounding water and by the soft parts of the animal. But taking into consideration that the skeletal elements had an average thickness of 0.2 mm and the head length was about 1.3 cm, we consider the pictures fairly good. A smaller focus in a heavier X-ray tube would doubtless improve the quality by increasing the focus-film distance.

More accurate measurements were obtained by injecting small lead particles into the animals, and by measuring the distances between these particles. Injected animals did not behave differently from non-injected animals. 2 lead particles were attached to the pertinax tube. From reference measurements of the neurocranium to these stable particles it can be shown that the whole animal did not move and that the animal was in a vertical fairly normal position. It is not necessary to use reference points, but it is easier to do so with complicated movements and 2 direction projections.

In the graphs (Figure 4) movements of 2 elements in lateral projection are plotted. Point Dm lies in the connective tissue close to the syndesmosis of the lower jaw bars. Thus the vertical movement represents the closing and opening of the mouth during respiration. The greatest value is the closed position. This movement can also be observed externally and measured on normal movie-pictures. It is taken here as a reference movement. Point L'm is situated on the rostral part of the tongue. The surprising fact is that the tongue appears to move almost only in a dorso-ventral direction. This is in contrast to the generally accepted view that there is con-

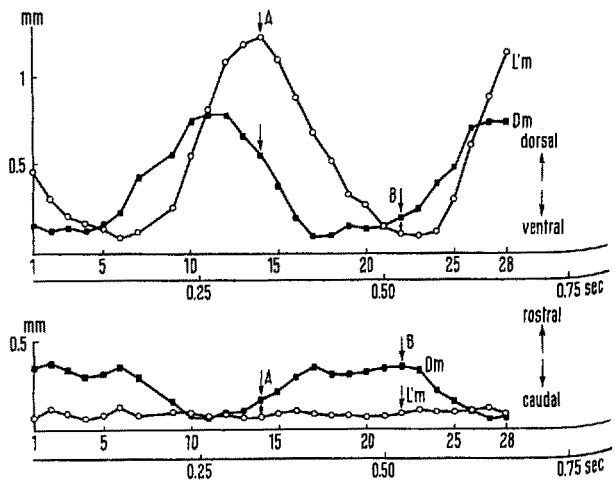

Fig. 4. Movement of 2 elements during the respiratory movement in *Gasterosteus aculeatus*. (A) and (B) refer to the radiographs of Figure 3.

siderable rostro-caudal tongue movement in teleost fishes[5-7].

The explanation of muscle activity and structure of joints will be published later, together with the results of vertical X-ray pictures. Here it suffices to conclude that this technique permits an easy determination of the movements of the parts in intact organisms. For the generally accepted conception of the double acting pump, those observations have some consequences. They strongly suggest that the respiratory movement must be considered to be at least partly a peristaltic movement[8].

Zusammenfassung. Ein einfacher Apparat ermöglicht mit direkter Röntgenbestrahlung kinematographische Aufnahmen von Tierbewegungen. Der Apparat besteht aus einer Drehscheibe und einer Säule von Zahnröntgenfilmen, welche durch die Scheibe jeweils nach Belichtung entfernt wird. Als Beispiel wurden Fisch-Atmungsbewegungen analysiert.

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Zoological Laboratory, State University, Leiden (The Netherlands), August 23, 1966.

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8 We are very thankful to J. Altink, W. Duk, F. J. S. W. Körner and A. L. C. Heemskerk for the construction of the apparatus and their critical and stimulating discussion during its design. We also thank C. Elzinga for preparing the drawings and Drs. C. Gans and H. J. de Jongh for their valuable correction of the English text.

Immunofluorescent Technique to Detect Anti-Penicillin Antibody

A number of tests with immunofluorescent methods have been performed in order to achieve a simple and quick technique for investigating immunological cross reactions between different antibiotic molecules.

We have obtained an easy indirect method, which we hope will resolve this kind of problem, in which the antibiotic molecule is fixed on rabbit erythrocytes to dispose of an antigen clearly visible through the microscope. Specific immunosera are mixed with them on smears and afterwards an anti-rabbit fluorescent-labelled serum is used.

The materials used in the biological test are: (a) *erythrocytes* from rabbits in which penicilolyl-polylysine molecules[1] have been fixed. These were prepared by mixing rabbit blood with an equal volume of modified Alsever's liquid[2]. After washing the erythrocytes several times in buffered saline solution, 1 ml was maintained with 9.0 ml of 10^{-6} *M* peniciloyl-polylysine solution (Cilligen), first at 37 °C for 30 min and then at 4 °C overnight. Later the erythrocytes were washed 6 times in buffered saline solution and were then ready for use.

(b) *Anti-drug immunosera.* These were obtained by inoculating rabbits with penicillin plus Freund complete adjuvant mixture[3] so that the rabbit received 125 mg penicillin plus 1 ml of adjuvant in a volume made up to 2 ml with saline. This volume was injected into the pad of each foot and at 4 points on the rabbit's back. During the first 4 weeks the animal received 2 weekly injections and then 2 more at 10 day intervals. Samples of blood from each rabbit were taken by heart puncture 10 days after the last injection and the antibody titre was found by hemagglutination of these sera against penicillin-coated erythrocytes[4]. When the titre was high enough, the rabbits were bled to death.

(c) *The goat anti-rabbit globulins* (provided by the Pasteur Institute). These were labelled with fluorescein isothiocyanate at a rate of 1/20.

The method we propose is as follows: a smear of peniciloyl-polylysine coated erythrocytes is fixed with acetone at − 20 °C for 10 min and then treated with anti-penicillin rabbit serum for 30 min at 37 °C. After washing in buffered saline solution, it is stained by fluorescent anti-rabbit reagent for 30 min at 37 °C and then washed again and mounted with buffered glycerine, pH 9.0, to be

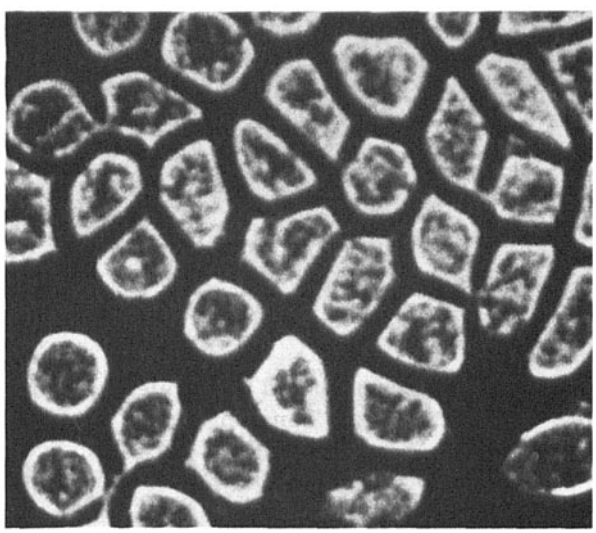

Figure a

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