translated into concentration of hemagglutinins on the standard curve.

The agglutination-sedimentation curves not only vary in slope as a function of the concentration of hemagglutinins; the point at which the curve starts to rise depends also on the concentration of the agglutinating virus, the more dilute it is the later the point at which the curve starts to rise (Table).

The hemagglutinins of 6 different viruses were estimated by this method: Newcastle disease virus (NDV), influenza A, encephalomyelocarditis (EMC), Semliki Forest virus (SFV), adenovirus 3 and polyoma. The method was found to be satisfactory for all the viruses tested except polyoma virus. Chick erythrocytes were employed in the test for NDV and influenza, sheep erythrocytes for EMC, duck erythrocytes for SFV, and rhesus erythrocytes for adenovirus 3.

Hemagglutination inhibiting antibodies may also be titrated with the help of the fragiligraph, by determining the antibody dilution which decreases the slope of the agglutination-sedimentation curve of a control virus to predetermined value.

Relationship between the form of the agglutination sedimentation curves and NDV concentration

| Virus concentration HA units | Time at which curve starts to rise[a], min | Slope |
|---|---|---|
| 27 | 13 | 1.0 |
| 55 | 4 | 2.0 |
| 110 | 3 | 4.8 |
| 220 | 1.8 | 8.8 |

[a] Time elapsed (in minutes) between mixing of virus and red cells and the point at which the curve starts to rise.

*Résumé.* Une méthode est décrite pour la mesure des hémagglutinines virales et des anticorps inhibiteurs des hémagglutinines au moyen d'un système photoélectrique et enregistreur automatique – le fragiligraphe. Une suspension des érythrocytes et du virus est introduite dans la microcuvette du fragiligraphe. L'agglutination et la sédimentation des érythrocytes causent l'augmentation de la transmission de la lumière à travers la suspension. Le temps jusqu'au début de l'inflexion de la courbe et son angle permettent d'évaluer la concentration du virus.

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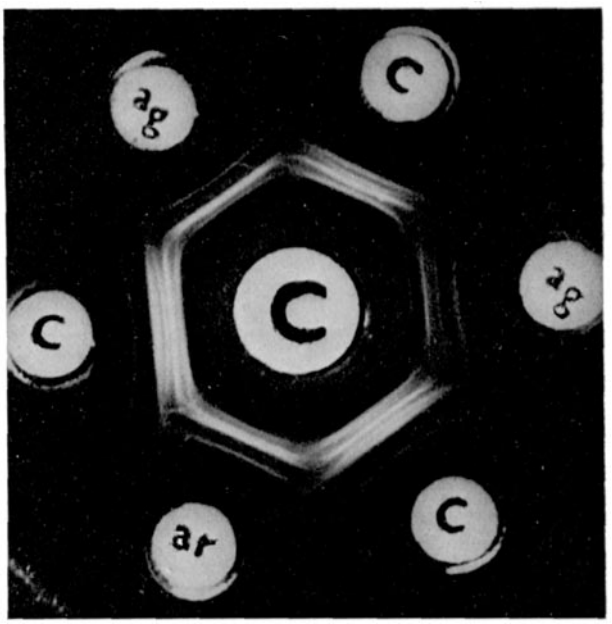
## The Agar Gel-Diffusion Technique as a Method of Differentiating Mosquito Larvae

The gel-diffusion technique has been previously used by us for the antigenic analysis of mosquito eggs (ZAMAN and CHELLAPPAH[1]). This is a report of the antigenic analysis of mosquitoes using 4th instar larvae. The three Culicines reared in our laboratory, *Culex pipiens fatigans, Aedes aegypti* and *Armigeres subalbatus* have been investigated.

The antigens were a saline extract of 4th instar larvae. Approximately 100 larvae were macerated in 1 ml isotonic saline in a glass tissue grinder. During the maceration the grinder was kept immersed in an ice-bath. The suspension was then left overnight at 5° C with a magnetic stirrer. Next morning, it was cleared by centrifugation and standardized to contain approximately 3 mg of proteins/ml.

The rabbits were immunized with 4th instar *Culex* antigen injected subcutaneously along with Freund's adjuvant (Difco) at weekly intervals. Each rabbit received approximately a total of 2000–3000 larvae in 8–10 doses. The rabbits were held one week after the last injection and the serum collected. The gel-diffusion plates were made with Ion agar No. 2 (Oxoid). The concentration of agar was 1% in distilled water. To this 0.01% Na merthiolate was added as a bacteriostatic agent. The reaction was allowed to take place at room temperature in a moist chamber. The antigens and the sera were used undiluted. The lines were photographed after 7–10 days.

The Figure shows the reaction obtained with *Culex* antiserum when used against *Culex, Armigeres* and *Aedes* antigens. With the homologous system a single distinct line was seen near the antigen well. No corresponding line was observed with *Aedes* and *Armigeres* antigens. The line




C, Central well = *Culex* antiserum. C, Outside wells = *Culex* antigen. ag = *Aedes* antigen. ar = *Armigeres* antigen.

[1] V. ZAMAN and W. T. CHELLAPPAH, Exper. *20*, 429 (1964).

was followed by a group of at least 5 more lines. All these lines were more distinct in the homologous system. However, a number of these lines showed a reaction of identity with the heterologous antigens.

Previously it was shown (ZAMAN and CHELLAPPAH[1]) that the antigens from eggs of *Culex* could be easily distinguished from those of *Aedes* and *Armigeres*, using the gel-diffusion technique. In this study using the 4th instar larvae of the same genera a much more complex picture is obtained. Although it was possible to distinguish *Culex* larvae from *Armigeres* and *Aedes*, there was a great deal of cross-reaction. In the case of larvae, unlike the eggs, some of the cross-reaction could be due to gut contents and other extraneous antigens. It is, therefore, suggested that for comparative studies clearer and more reliable results are likely to be obtained with egg antigens as compared to larvae.

*Zusammenfassung.* Mit der Gel-Diffusionstechnik lassen sich die Larven von *Culex pipiens fatigans, Aedes aegypti und Armigeres subalbatus* unterscheiden. Wegen teilweise gemeinsamen Antigenen von *Armigeres subalbatus* und *Aedes aegypti* ist eine Differenzierung dieser verschiedenen Species bei Verwendung von Larven weniger eindeutig als bei Verwendung von Eiern.

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## STUDIORUM PROGRESSUS

### The Isolation and Biosynthesis of Highly Polar Steroids Found in Placental Tissue

In 1960 it was demonstrated by ULSTROM et al.[1] that C-6 oxygenated steroids were present in neonatal urine, while FRANZ et al.[2] produced evidence that during pregnancy the urinary concentrations of the polar steroid 6$\beta$-hydroxycortisol is elevated above the normal non-pregnant state.

Recent work in this department (DIXON and PENNINGTON[3]) has identified the presence of the 20$\alpha$-hydroxy derivative of 6$\beta$-hydroxycortisol (Compound 4c), the 20$\beta$-hydroxy derivative of 6$\beta$-hydroxycortisol (Compound 4d) and the 20$\beta$-hydroxy derivative of 6$\beta$-hydroxycortisone (Compound 3) in late pregnancy urine. A further polar steroid (Compound 4b) was also recognized, but so far has not been conclusively identified.

The source of highly polar steroids identified in pregnancy urine is uncertain and it was therefore of interest to investigate to what extent the placenta might possibly contribute to their production or alternatively provide a means of concentrating and storing some of them.

*Materials and methods.* Tissue: Human placentas were obtained immediately following normal full-term spontaneous delivery. These were frozen to $-14°C$ in plastic bags and maintained at this temperature until extraction was carried out.

Extraction of polar steroids: 2.5 kg of placental tissue was cut up into small pieces in the frozen state and minced by means of a mechanical meat grinder ('Kenwood', Surrey, England). The mince was extracted with 2 l of freshly redistilled ethyl acetate for 4 h at 40°C. The ethyl acetate extract was decanted off and filtered through muslin in order to remove large meat particles. A further 1 l of ethyl acetate was then added to the placental residue and extraction continued for a further 2 h at 40°C.

The combined ethyl acetate extracts, which were now bright yellow in colour were evaporated under vacuum at 37°C to a final volume of 200 ml. 400 ml of *n*-heptane were added to the ethyl acetate extract and the solution extracted with 200 ml of distilled water on three occasions. In this process much of the contaminating non-polar material is left in the organic phase and the highly polar corticosteroids pass almost quantitatively into the aqueous phase (FRANZ, KATZ, and JAILER[4]). To the aqueous extract was added 20% (w/v) anhydrous sodium sulphate and the solution was extracted with 300 ml of ethyl acetate on two occasions. The ethyl acetate was washed with two 50 ml portions of a solution of 10% (w/v) sodium carbonate and 20% (w/v) anhydrous sodium sulphate in water. (Sodium carbonate was used in preference to sodium hydroxide for washing, since only very low yields of polar steroids were expected from the placental extract and VENNING[5] had presented evidence that there can be destruction of small amounts of adrenal steroids by alkali.) The washed extract was dried over anhydrous sodium sulphate and evaporated down under vacuum at 37°C. The residue was transferred to a test tube with small volumes of chloroform:methanol (1:1) and finally with pure methanol. The solvent was then evaporated off under a stream of nitrogen at 37°C.

The residue dissolved in chloroform:methanol (1:1), was transferred to Whatman 3 mm paper as a thin band 8 cm wide. After equilibration for 3 h in benzene:methanol:water 2:1:1 by vol (BUSH[6]) the chromatogram was developed for $5\frac{1}{2}$ h. On examination under UV-light at 254 m$\mu$, four UV absorbing zones were observed; the most intense of these zones was at the origin. A 2 mm strip from the origin to the solvent front was cut out from the centre of the 8 cm strip and dipped through a 2:1 solution of 10% aqueous sodium hydroxide in 50% methanol and 0.025% blue tetrazolium in ethyl alcohol (DIXON[7]). After heating at 60°C for 10 min, four yellow fluorescing zones were observed under UV-light at 365 m$\mu$, each corresponding to an area which had shown UV absorption. Since it was only of interest to investigate the most polar area, the zone of the origin was subjected to further investigation. No further identification procedures were carried out on the other three zones. An area extending 2 cm each side

[1] R. A. ULSTROM, E. COLLE, J. BURLEY, and R. J. GUNVILLE, J. clin. Endocrin. Metab. *20*, 1080 (1960).
[2] A. G. FRANTZ, F. H. KATZ, and J. W. JAILER, Proc. Soc. exp. Biol. Med. *105*, 41 (1960).
[3] W. R. DIXON and G. W. PENNINGTON, to be published.
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[5] E. VENNING, Recent Progr. Hormone Res. *9*, 300 (1954).
[6] I. E. BUSH and V. B. MAHESH, Biochem. J. *71*, 705 (1959).
[7] P. DIXON, personal communication (1961).