workers have reported brief axenic maintenance of the monogononts *Brachionus variabilis*[3,4] and *Lecane inermis*[4] and of the bdelloid *Philodina acuticornis* var. *odiosa*[4], but, for the most part, these organisms have merely survived without appreciable development. We are now able to report conditions whereunder the continuous cultivation of *L. inermis* has been realized.

During a period of almost three years numerous media, based on complex preparations, variously of vertebrate and bacterial origin, were tried, but failed to lead to axenic cultivation—first for *B. variabilis* and lately for *L. inermis* and *P. acuticornis*. The media in which, by contrast, *L. inermis* has now grown and reproduced for successive generations in serial subculture contain, as the crucial ingredient, a heated (lamb) liver extract (HLE) prepared according to a strict recipe[5] and sterilized by passage through an ultrafilter (Millipore).

The axenic *L. inermis* currently maintained by us descend from cultures originally established with rotifers that had been reared monoxenically[4] with an unidentified species of Gram-negative, rod-shaped bacteria. In the initial successful experiment, axenizing was accomplished by our selecting several dozen individuals with micropipettes, passing them through one rinse of 0.5 ml pond water previously sterilized by ultrafiltration (twice through sintered glass), and leaving them for approximately 2 h in ca. 20 ml distilled water containing, per ml, 1000 u K · penicillin G and 100 γ streptomycin · SO_4. Individuals were then transferred through one further sterile pond water rinse into 0.1% Horlick's malted milk, pending inoculation into small test tubes (10 mm outer diameter), each containing 0.2 ml medium to be tested. Each tube received a single rotifer and thereafter was tightly stoppered. Except (presumably) where injured or past reproductive age, such isolated individuals inoculated into suitable media became the progenitors of cultures consisting, at their acme, of some hundreds of active rotifers. Subsequently, subcultures have been readily initiated with single individuals from thriving axenic populations. Indefinite maintenance, in axenic culture, of *L. inermis* thus appears possible, just as has been demonstrated for several rhabditid nematodes[6], of which *Caenorhabditis briggsae*, now six years in continuous axenic cultivation in cell-free, liquid media, has been longest maintained.

HLE is not tolerated by *L. inermis* at $^1/_4$ dilution (the most concentrated tested), and only moderately well at $^1/_8$. At $^1/_{16}$ dilution growth is excellent. Initially the HLE was doubly supplemented with a 'GM' vitamin mix (at 1% the level used for *C. briggsae*[7]) plus Horlick's malted milk (at 0.075%); but current experiments demonstrate that HLE alone suffices.

Success in the axenic cultivation of *L. inermis* now opens the way to the development of chemically defined (holidic[8]) media for it, following the general pattern already set by studies with *C. briggsae*, for which a considerable advance toward such a medium has been realized[7]. These and similar organisms, grown axenically in holidic media, should in time become elegant tools with which to probe fundamental biological problems—particularly those depending for solutions upon the penetrating insights that comparative animal biochemistry and nutrition, as mature and mobilized disciplines, will inevitably provide.

Résumé. Le Rotifère monogononte *Lecane inermis*, a été mis par les auteurs en cultures axéniques capables de sous-cultivation indéfinie. C'est le premier Rotifère soumis à cette expérience.

E. C. DOUGHERTY[9], B. SOLBERG, and D. JOANNE FERRAL

Laboratory for Gnotobiotic Studies, Berkeley (California), October 22, 1960.

[3] H. A. NATHAN and A. D. LADERMAN, Ann. N. Y. Acad. Sci. *77*, 96 (1959).

[4] E. C. DOUGHERTY, B. SOLBERG, and L. G. HARRIS, Anat. Rec. *137*, 350 (1960); Science *132*, 416 (1960).

[5] Originally developed by Dr. F. W. SAYRE (Laboratory of Comparative Biology) for studies on the nutrition of the nematode *Caenorhabditis briggsae* and generally prepared as follows: a liver from a freshly slaughtered lamb is allowed to age at 4°C for 24 h, then homogenized in a Waring Blendor with equal parts, w./v., distilled water, and thereafter heated in 100 ml amounts to 53°C in a waterbath and maintained at that temperature, with constant stirring, for exactly 6 min; next the product is centrifuged in the cold at 20000 G, and the supernatant (HLE) decanted and ultrafiltered immediately, or frozen pending filtration; filter d or unfiltered, it is stored at −17°C. The use of HLE in thestudy of nematode nutrition has not yet been reported in published form.

[6] W. L. NICHOLAS, E. C. DOUGHERTY, and E. L. HANSEN, Ann. N. Y. Acad. Sci. *77*, 218 (1959).

[7] E. C. DOUGHERTY, E. L. HANSEN, W. L. NICHOLAS, J. A. MOLLETT, and E. A. YARWOOD, Ann. N. Y. Acad. Sci. *77*, 176 (1959).

[8] E. C. DOUGHERTY, Ann. N. Y. Acad. Sci. *77*, 51 (1959); in J. N. SASSER and W. R. JENKINS (Eds.), *Nematology, Fundamentals and Recent Advances with Emphasis on Plant Parasitic and Soil Forms* (Univ. N. Carolina Press, Chapel Hill 1960), p. 291.

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PRO EXPERIMENTIS

On the *in vitro* Bioassay for Measuring Melanophoretic Activity

This report will describe several modifications of the *in vitro* frog skin bioassay of SHIZUME et al.[1] as used in this laboratory for the estimation of melanophoretic activity. Particular attention has been directed toward the development of a 4-point assay procedure which can be easily controlled by classical mathematical analysis[2].

Materials and Methods. Pieces of skin of the legs and thighs of *Rana pipiens* frogs which have been kept in constant light for at least 2 weeks are removed, trimmed, and placed in concentric rings in a manner similar to that described previously[1]. Anodization of the aluminum parts of these rings was found to prevent completely the corrosion which occurs normally on the untreated aluminum parts when immersed in the electrolyte solutions. Anodized rings have been used here for more than 2 years without evidence of corrosion.

Variations in successive reflectance measurements on fragments of skin during control periods were encountered in early work with the assay; these were due essentially to disorientation of the skin fragment for each reading from the position in which it was first placed for the original reading. To remedy this, the bakelite outer rings[1] were designed with four equidistant V-shaped notches in the base to correspond to four ridges which we had sealed by fusion in the bottoms of 50 ml pyrex beakers. These ridges extend from the edge of the beaker about a third of the way to the center. A clear area remains in the center of the beaker through which the reflectance readings are taken. The same apparatus as employed by SHIZUME et al. (Photovolt Photoelectric Reflection Meter Model 610, Photovolt Corporation, New York) is used to indicate changes in reflectance of the skins. The use of these 'inter-

[1] K. SHIZUME, A. B. LERNER, and T. B. FITZPATRICK, Endocrinology *54*, 553 (1954).

[2] C. I. BLISS, *The Statistics of Bio-Assay* (Academic Press, Inc., Publ. New York 1952), 1 vol.

Beaker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sample	LL_1	RT_1	LT_1	RL_1	RL_2	LT_2	RT_2	LL_2	LL_3	RT_3	LT_3	RL_3	RL_4	LT_4	RT_4	LL_4
Addition	S_1	S_2	U_1	U_2	S_1	S_2	U_1	U_2	S_2	S_1	U_2	U_1	S_2	S_1	U_2	U_1

locking' rings and beakers has eliminated the variation to such a degree that a skin may be removed from and then replaced in a beaker with changes of no more than 0.5 to 1.0 reflectance unit in the successive readings. A mark made on the base of each beaker is lined up with a mark on the head of the search unit for each reading, to insure that the position of a skin over the field of light will not vary. A 250 ml beaker darkened inside with flat black paint is placed on the top of the search unit over the samples as they are read in order to eliminate the effect of extraneous light. With these precautions constant readings of control skins over a period of time may be observed. No filters are used and the measurements are made with the white light from the bulb of the search unit, the galvanometer set to 100 with the grey enamel standard No. 2 furnished with the Reflection Meter.

The MSH-standard used is an aliquot of the original preparation reported by SHIZUME et al.[1]. It has a melanophoretic activity of 2.5×10^4 units/mg by definition, 1 unit equivalent to 0.04 µg. It is diluted to concentrations of 0.4 µg/ml, and 0.133 µg/ml to be used as high (S_2) and low (S_1) standards corresponding to 10.00 and 3.33 MSH units/ml respectively. The frog Ringer's solution (NaCl 6.50 g/l, KCl 0.14 g/l, $CaCl_2$ g/l, $NaHCO_3$ 0.20 g/l) in which all standards or unknowns are diluted is prepared using de-ionized (Crystalab Deeminizer–Dynalab Corporation) distilled water having less than one part per million of electrolyte. Standards and unknowns are always added in a volume of 1.0 ml to the skins which are maintained in 20.0 ml of the Ringer solution. To insure mixing of materials added, the solution is stirred and bubbled with the pipette as the 1.0 ml aliquot is introduced. During the period in which the melanophoretic response is achieved, the beakers containing the skins are incubated in a 22–23°C water bath, optimal temperature for the response having been described as 20–25°C[1].

The design of both the 4-point and bracketed assays in this laboratory utilize 16 specimens of skin, and thus 4 frogs. The samples of skin are removed and placed in a specific order in beakers numbered from 1 to 16, as indicated in the Table so that each group of S and U will have an homogenous population (as to origin) of skin fragments.

In this arrangement LL signifies left leg, RT indicates right thigh, etc., and the subscript number identifies the frog from which the skin was taken. S_1, S_2, U_1, and U_2 represent the low and high standards and unknowns. It should be noted from their distribution in the Table that there will be one response for each skin from each of the frogs used, and that every standard and unknown is applied to a LL, RT, LT, and RL. Thus in a 4-point assay, we have a total of 16 observations, i.e. 4 observations per dose of U_1, U_2, S_1, S_2. In a bracketed assay the skins used for U_1 and U_2 may be substituted in use for as many as 8 different unknowns. The measurements are made as follows:

Y_1: Is the reading of the reflectance of the skins after they have been allowed to lighten and equilibrate in frog Ringer for 60 min. (The solution has been changed once during this hour.) When Y_1 is recorded, 1.0 ml of S_2 (0.4 µg/ml; 10 MSH units) is added to *each* beaker.

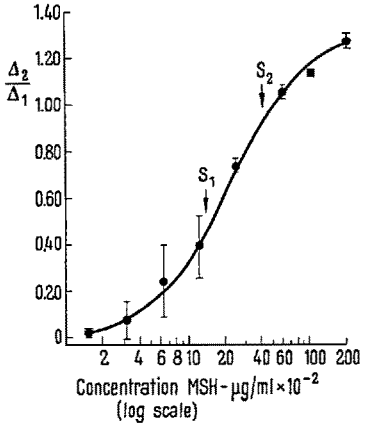
Y_2: Is the reading taken 60 min after the additions made at Y_1. During this hour the skins will have approached a maximal response to the material added. Immediately following this reading the Ringer solution over all skins is changed. The samples are again allowed an hour period to lighten during which the solution is changed once.

Y_3: Is the reading taken then: it is followed by the addition of both standards and unknowns as described in the Table. At this time the Y_3 reading should be checked against the initial reading Y_1, and discard made of skins not returning to within 5 reflectance units of the first (Y_1) reading.

Y_4: Is the final reading and is taken 60 min after the additions made at Y_3.

The difference between the first two readings ($Y_1 - Y_2$) is designated as Δ_1, and that between the second two ($Y_3 - Y_4$) as Δ_2. The ratio of Δ_2/Δ_1 is determined for each of the 16 responses, and a standard curve is plotted on semi-log graph paper using the mean values obtained for S_1 and S_2. The Δ_2/Δ_1 readings for the unknowns may then be applied to the graph and computed into terms of MSH units. The curves for S and U can also be calculated mathematically[2].

A log dose-response evaluation of the MSH standard was made and the sigmoid curve shown in the Figure obtained. The mean responses shown with their standard errors were achieved using a log-interval of 2 and duplicate observations (i.e. 2 skins per dose). It can be seen that the 0.4 µg/ml and 0.133 µg/ml values chosen to be used for S_2 and S_1 in all subsequent assays fall within the linear portion of the curve.



Dose-response curve for MSH reference standard in the *in vitro* assay

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[4] A. V. SCHALLY, R. N. ANDERSEN, J. M. LONG, and R. GUILLEMIN, Proc. Soc. exp. Biol. Med. *104*, 290 (1960).

[5] R. GUILLEMIN and W. KRIVOY, C. R. Acad. Sci. (Paris) *250*, 1117 (1960).

[6] R. GUILLEMIN, A. V. SCHALLY, R. N. ANDERSEN, H. S. LIPSCOMB, and J. M. LONG, C. R. Acad. Sci. (Paris) *250*, 4462 (1960).

[7] *Acknowledgments.* This work was supported by research grants (No. A-2543, C_1, C_2) from the National Institute of Health (NIH) of the U.S. Public Health Service and Contract No. AF 419657)-224 with the School of Aviation Medicine, U.S. Air Force, Wright Air Development Division, Lackland Air Force Base, Texas.

Statistical analysis of the dose-response relationship of the S_1 and S_2 standards from 22 individual assays has been made. In 17 of these assays there were 4 responses for each of the standards, in 3 assays there were three, and in 2 assays only two responses per dose of S_1 and S_2. Results of the analysis are as follows: Composite slope $b_c \pm$ S.E. = 1.41 $\pm$ 0.14; mean $\lambda = s/b \pm$ S.E. = 0.247 $\pm$ 0.0273; variance s between slopes = not significant at $p = 0.005$. The spread of samples was fairly large about the composite line, but much less so in individual assays. There was no significant difference between the slopes of the 22 assays when considered as a group. The constancy of the slope between assays would indicate that the composite curve may be used as a nomogram to evaluate relative activity between several samples assayed in one experiment. Classical 4-point assays with a set of standards should always be run if an accurate absolute value in MSH units/mg is desired on any given material.

This assay has been used extensively in this laboratory over the last three years to our satisfaction for determining the specific MSH activity of given materials and to follow with great accuracy purification procedures on various starting materials containing α-MSH, β-MSH or related peptides[3–6].

Résumé. Les auteurs décrivent diverses modifications au test original de SHIZUME et al. pour mesurer l'activité mélanophorétique *in vitro*. Une analyse mathématique des résultats obtenus avec cet étalonnage en montre les caractéristiques satisfaisantes.

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PRO EXPERIMENTIS

Quelques commentaires sur les méthodes de culture *in vitro* de jeunes blastodermes de poulet[1]

La possibilité d'opérer de jeunes blastodermes d'oiseaux non dans l'œuf, mais en culture *in vitro*, offre des avantages évidents et de longue date diverses techniques sont employées dans ce but. Le procédé courant consiste à élever le blastoderme sur un substratum nutritif ferme et fibreux. WADDINGTON[2] utilisait du plasma sanguin de poule et du jus embryonnaire coagulés. Depuis les investigations de SPRATT[3] on ajoute de la gélose au milieu nutritif proprement dit pour lui conférer une certaine consistance. La composition de ce milieu diffère largement selon les auteurs et les buts de leurs recherches. D'après SPRATT l'albumen seul, ou mélangé au vitellus, satisfait le mieux aux besoins nutritifs de l'embryon, tandis que WOLFF et SIMON[4] préconisent l'emploi de jus embryonnaire. Dans ces conditions de culture le développement du corps embryonnaire peut se poursuivre normalement, surtout si le blastoderme a été étalé sur le substratum après la formation des ébauches axiales. Cependant, l'extension périphérique du blastoderme est toujours arrêtée.

En 1955 NEW[5] a démontré que cette extension est due à l'adhérence du bord d'enveloppement à la membrane vitelline laquelle doit être légèrement tendue. Le bord d'enveloppement, en s'élargissant grâce à la prolifération active de ses cellules, et en glissant sur la membrane vitelline, étire littéralement le blastoderme tout entier. Partant de ces observations, NEW a élaboré une technique de culture *in vitro* beaucoup plus proche des conditions du développement normal. On immerge le jaune d'œuf dans une solution physiologique; on découpe la membrane vitelline autour du blastoderme selon le plan équatorial qui lui correspond. Le cercle de la membrane vitelline, détaché du vitellus, est étalé, le blastoderme en haut, sur un verre de montre et alors on pose un anneau de verre sur la membrane vitelline. Ensuite la solution physiologique est déversée du verre de montre et une petite quantité d'albumen est introduite sous la membrane vitelline. Pour l'incubation l'ensemble est mis dans une chambre humide. Dans ces conditions aussi bien le développement et la croissance du corps embryonnaire que l'extension périphérique du blastoderme se poursuivent normalement jusqu'au moment où l'embryon atteint le stade auquel, s'il était dans l'œuf, il aurait commencé à s'enfoncer dans le vitellus.

Rappelons encore que, comme NEW[6] l'a démontré, le blastoderme absorbe l'eau de l'albumen ce qui permet à son feuillet interne de sécréter abondamment le liquide contenu normalement dans la cavité sous-germinale. Contrairement donc aux cultures, où le blastoderme est situé à l'interface du substratum nutritif et de l'air saturé de vapeur d'eau, les blastodermes élevés selon la technique de NEW se développent dans un milieu liquide normal. Une petite quantité de ce liquide s'accumule entre le blastoderme et la membrane vitelline, en formant une mince couche limitée périphériquement par le bord d'enveloppement qui adhère étroitement à la membrane, tandis que le liquide sécrété par l'entophylle le submerge d'une couche de plus en plus épaisse.

La méthode de NEW, si satisfaisante soit-elle, présente, néanmoins, un certain désavantage: elle ne permet guère de pratiquer sur les blastodermes cultivés de multiples interventions expérimentales lesquelles doivent être faites du côté dorsal. Si l'on détache le blastoderme pour le remettre en sens inverse sur la membrane, le bord d'enveloppement, grâce à l'affinité de sa face externe pour la membrane vitelline, s'enroule et après quelque temps le blastoderme prend la forme d'une vésicule. Récemment, NEW[7] a essayé d'étaler le blastoderme, sa face ventrale en bas, sur la face externe de la membrane vitelline. Dans cette position, le bord d'enveloppement n'adhère pas à la membrane et ni son enroulement ni extension périphérique du blastoderme n'ont lieu. Signalons à ce propos un fait que NEW semble ignorer: la membrane qui englobe la sphère vitelline d'un œuf pondu est en réalité composée de la membrane vitelline proprement dite et d'une mince couche d'albumen extrêmement visqueux, le même qui forme les chalazes. Si ce n'était pas le cas, leur fixation sur le globe vitellin aurait été incompréhensible. D'autre part, pendant le passage du vitellus dans la trompe, de petites hémorragies se produisent fréquemment et alors des caillots de sang se déposent sur la membrane vitelline. Or, les coupes histologiques de cette dernière, prélevée sur un œuf pondu, montrent que ces caillots, pourvu qu'ils soient suffisamment minces, sont englobés entièrement dans les couches superficielles de la «membrane vitelline».

Afin de trouver une technique de culture permettant d'opérer aisément des blastodermes du côté dorsal et garantissant simultanément leur extension périphérique

[1] Travail subventionné par le Fonds national suisse de la Recherche scientifique.

[2] C. H. WADDINGTON, Phil. trans. Roy. Soc. B. *221*, 179 (1932).

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[4] ET. WOLFF et D. SIMON, C. R. Acad. Sci. *241*, 1994 (1955).

[5] D. A. J. NEW, J. Embryol. exp. Morph. *3*, 326 (1955).

[6] D. A. J. NEW, J. Embryol. exp. Morph. *4*, 221 (1956).

[7] D. A. J. NEW, J. Embryol. exp. Morph. *7*, 146 (1959).