STUDIORUM PROGRESSUS

Editorial note. On June 9, 1971, Professor Emil Witschi died at the age of 81 years. As a young man, Witschi started his scientific career with the epochmaking experimental work on the sexuality of amphibians in Basle, a work which he continued and completed 'extra muros' in America. His latest scientific publication, which appears once again in Basle, represents a return home for this exceptional embryologist, cytologist, geneticist and sex researcher, both in the literal and in the wider sense. H.M.

Developmental Causes of Malformation

Research in the etiology of human congenital malformations gained great impetus and new directions with the discovery of the sex-chromatin-bodies in 1949[1] and the chromosomal aneuploidies in 1959[2–4]. Since the latter often are found in association with abnormal individual traits some relationship between chromosomal patterns and pathologic syndromes is obvious. As long as only few aneuploidies had been observed, there was a tendency to assume that chance-nondisjunction or similar mitotic errors might be the very cause of these malformations. But such interpretations became untenable when chromosome abnormalities were found at very high frequencies in early aborted embryos[5,6] (Table I). CARR[7] and SINGH and CARR[8], in a material of 228 embryos and fetuses at ages up to the 24th week, found a total of 22% chromosome abnormalities, but 54 and 48.6% in 22 ruptured and 37 intact sacs without embryos, gestationally the youngest groups. Evidently, abortuses are a highly selected lot; but NISHIMURA[9] reports 3% aberrations in 100 karyotyped embryos from healthy pregnancies interrupted at 2 to 3 months, which is at least 6 times the frequency of survivers, i.e. live-born babies. His figure appears slightly high, considering that the early excessive rate of 'reproductive wastage' abates at about 6 weeks. By this time it amounts already to 66% as indicated by the study of reproduction in 210 couples, which had been selected by HERTIG and collaborators for proven fertility of both mates[10,11].

We are utterly ignorant of the ways and the means that control the steadily progressing degeneration of millions of ovocytes in the ovaries of the human female between birth and age 45, and yet selectively spare about 400 that may be released intermittently during 30 fertile years[12]. However in consideration of this mass destruction, it is likely that some of those ovulated have suffered partial damage and contribute to the class of 'bad eggs' – to use an expression by HERTIG.

Analysis of the mechanics of partial or complete deterioration of pre- and post-ovulatory human eggs is particularly difficult because no planned experimental investigations are possible. Work with other mammals provides valuable results[11], though it is handicapped by lack of clear controls. All mammalian species have high rates of reproductive wastage. Consequently spontaneous aberrations occur in the control lots. The evolution of nurture necessitated a reduction of the number of offspring. For clear experimentation one must turn to more primitive vertebrate species with full fertility. In most frogs of the genus *Rana* virtually all ovulated eggs (usually several thousand at one mating) are fertilizable and capable of normal development. If stored at low room temperature (18–19 °C for *Rana temporaria*) full fertility is maintained

[1] M. L. BARR and E. G. BERTRAM, Nature, Lond. *163*, 676 (1949).
[2] J. LEJEUNE, M. GAUTHIER and R. TURPIN, C. r. Acad. Sci., Paris *248*, 1721 (1959).
[3] P. A. JACOBS and J. A. STRONG, Nature, Lond. *183*, 302 (1959).
[4] C. E. FORD, K. W. JONES, P. E. POLANI, J. C. C. ALMEIDA and J. H. BRIGGS, Lancet *1*, 711 (1959).
[5] K. MIKAMO, A. J. Obstet. Gynec. *106*, 243 (1970).
[6] J. G. BOUÉ et A. BOUÉ, Presse méd. *78*, 635 (1970); and pers. communication.
[7] D. H. CARR, Obstet. Gynec. *26*, 308 (1965).
[8] R. P. SINGH and D. H. CARR, Obstet. Gynec. *29*, 806 (1967).
[9] H. NISHIMURA, Proc. 6th World Congress Fertility and Sterility, Tel Aviv, 1968 (Isr. Acad. Sci. and Humanities; Jerusalem 1970), p. 106.
[10] A. T. HERTIG, *Comparative Aspects of Reproductive Failure* (Ed. K. BENIRSCHKE; Springer, New York 1967), p. 11.
[11] E. WITSCHI, *Congenital Malformations* (Eds. F. C. FRASER and V. A. MCKUSICK; Excerpta Med., Amsterdam and New York 1970), p. 157.
[12] E. WITSCHI, Int. Acad. Pathol., Monograph 3 (Eds. H. C. GRADY and D. E. SMITH; Williams and Wilkins, Baltimore 1963), p. 1.

Table I. Frequencies of abnormal karyotypes (%) at early misdevelopment: Neurulae (Stages 13–17) and tailbud embryos (Stages 18–33)

Material	Stage	Age	Number	%	Stage	Age	Number	%
Human[5]	–17	–4w	8	75	18–33	5–8w	18	50
Human[6]	–17	–4w	379	67	18–33	5–8w	309	58
Anuran[15]	17–18	3–4d	48	87				

The Human materials were karyotyped abortuses. The anurans were malformed specimens cultured from overripe eggs and selected at stages 17 to 18 for chromosome analysis. 40 embryos from control groups had normal karyotypes.

Table II. Distribution of karyotypes in a group of 48 malformed embryos (Stages 17–18) of *Rana pipiens* and *Xenopus laevis*

Karyotype:	Haploidy	Monosomy	Diploidy	Trisomy	Triploidy	Tetraploidy	Mosaicism
Number	2	11	6	4	3	1	21
Percent	4.2	23	12.5	8.3	6.25	2.1	43.75

for 3 days. Later deterioration leads to overripeness, ending in death after the 5th day. It has long been known that delayed fertilization of frog eggs produces all major types of monstrosities observed in human fetuses[13]. More recently it was also shown that in *Xenopus* an extended retention of eggs in ovarian follicles (delayed ovulation) results in overripeness[14]. While the *Xenopus* type offers no possibility of exact controls – the degree of overripeness must be estimated from the severity of misdevelopment – it serves in the study of effects of age and of the time sequences in follicle ripening.

The amphibian material furnishes also experimental proof that overripeness produces the chromosomal anomalies that are observed in the human[15]. Consulting Table I shows a particularly high frequency of aneuploidy in amphibian monsters at stages corresponding to human embryos of 4 to $4^1/_2$ weeks. This is not surprising and in fact might even be expected since all were malformed products of experimental overripeness, while some in the human collections were morphologicly (and mostly also chromosomally) normal embryos, possibly ejected from causes such as attempted induced abortions. It is noteworthy that among amphibian monsters, as in the youngest of human defectives, there remains a considerable number with normal karyotype and that the aneuploid aberrations are of similar diversity of character as in man (Table II). The high frequency of chromosomal mosaics (44%) is not surprising. Overripeness is primarily a degradation of the plasma and direct observation of frog eggs and embryos shows that often not all parts of a germ are evenly affected[13, 16]. The mosaics of this particular study seem to have originated from nondisjunction during first or second cleavage divisions, though other possibilites, like loss of chromosomes and double fertilization, are not excluded.

Overripeness of the egg is not only cause of chromosome disarrangement. Some of the 45 *XO* and, of course, all 47 *XYY* cases can be traced to paternal sources[17]. Nondisjunction of the *YY* diad in the second miotic division of spermatocytes should result in equal numbers of sperms without any, or with two *Y* chromosomes. Since eggs which they fertilize may be of 'good' quality in the ordinary proportion, both, *XO* and *XYY* embryos of such provenience, should have relatively fair chances of survival. The high mortality and teratoid morphology of *XO* embryos and fetuses of the 2nd and 3rd months[6] can be assumed to eliminate prevailingly cases with a paternal *X* – the loss of the maternal sex chromosome already being part of the egg-overripeness syndrome. On the other hand, loss of a *Y* in cleavage, as obviously happens in the history of monozygotic *XY–XO* twins, very probably results from the overripeness of the zygote.

In the years prior to 1959 it was still possible to think that all chromatin positive Klinefelters and hermaphrodites were sex reversed genetic femals. The author proposed that the shift toward maleness probably was due to overripeness of the egg[18]. This argument, based on experiments with amphibians, was temporarily placed in question through the discovery of additional *Y*-chromosomes in most chromatin positive Klinefelters and hermaphrodites[3, 4]. However, more recent findings of 46 *XX* males (25) and hermaphrodites (59), together with the puzzling cases of *XY* and *XX/XY* hermaphrodites (27)[19], the extreme variability of *XO* 'females'[20] and the numerous sex chromosome mosaics which defy attempts at disentangling on a purely cytogenetic basis[21] necessitate a return to the consideration of developmental factors and processes involved in sex differentation and sex reversal. The following are most important facts and tenents[22]:

1. A plasmatic, maternally inherited substance acts as germ cell determiner[23]. It can be partly or fully destroyed surgically, by radiation and by chemical deterioration in overripeness.

2. Sex differentiation of the gonads is induced exclusively by somatic elements of two gonadal territories: cortex, the ovary inductor and medulla, the testis inductor.

3. The alternative of developmental differentiation – cortical or medullary – is normally decided by the genetic constitution; but in case of inhibition or deterioration of one inductor system, the other may gain the lead (compensatory hypertrophy).

4. Important for sex development is the commonly observed reduction in number and vitality of the primordial germ cells, after overripeness of an egg. Their insufficient and late colonisation of the cortex entrains its poor development, which in turn enhances the possibility of compensatory medullary development. As a consequence testicular or intersexual gonads can form in embryos carrying only *X* chromosomes as well as any combination of *X* and *Y* chromosomes[11, 18].

5. Overripeness of the egg affects mainly the cytoplasm. Immediate and subsequent morphologic and physiologic changes suggest degradation of the maternally inherited and chromosome-derived proteins and enzymes, which make up the specific organization of the mature ovocyte[13].

6. Beginning liquefaction of achromatic fibers of the polar spindles leads to irregular distribution of chromosomes. Later the spindles dissolve fully and the chromosomes disperse or clump together.

Sixty years of discussions on theories of quantitative sex determination have led many cytogeneticists to think of sex chromosomes and certain autosomal complexes in terms of fixed values for maleness and femaleness. But even the mere fact that 3a + 2x drosophilae fluctuate from near female to near male intersexual types proves that physiologic conditions can modify the manifestation of genes. The activation of the sex genes depends on highly labile environmental factors. For *Xenopus* larvae the time of cortical (female) or medullary (male) differentiation arises during the 3rd week of development[22]. The gene activation and the long chains of the postgenetic processes can only be described in general terms. They consist of molecular reactions in which hormones that start DNA transcription are followed by the dispatch of RNA messengers, interaction with ribosomes, synthesis of polypeptides, proteins and enzymes. Interference by intra- and extracellular conditions is possible at every step. Overripeness produces infertility probably by affecting the germ line substance. More generally it becomes teratogenic by degrading and finally destroy ng the architecture, the stores of chemical precursors and the course of metabolism of the egg cell[11, 13].

[13] E. WITSCHI, Cancer Res. *12*, 763 (1952).
[14] K. MIKAMO, Cytogenetics *7*, 212 (1968).
[15] E. WITSCHI and R. LAGUENS, Devl. Biol. *7*, 605 (1963).
[16] E. WITSCHI, Arch. Mikrosk. Anat. *102*, 168 (1924).
[17] R. R. RACE, Phil. Trans. R. Soc. Lond. B *259*, 37 (1970).
[18] E. WITSCHI, Trans. 3rd Conference on Gestation (Jos. Macy jr. Found.; Ed. C. A. VILLEE, Princeton, N.J. 1956), p. 119.
[19] P. E. POLANI, *Congenital Malformations* (Eds. F. C. FRASER and V. A. MCKUSICK; Excerpta Med., Amsterdam and New York 1970), p. 233.
[20] G. A. HAUSER, *Intersexuality* (Ed. C. OVERZIER; Academic Press, London, New York 1963), p. 298.
[21] S. MAKINO, Meth. Teratol. Stud. Exp. Anim. Man, Kyoto 1968 (Eds. H. NISHIMURA and J. R. MILLER; Igaku Shoin, Tokyo 1969), p. 50.
[22] E. WITSCHI, *The Biochemistry of Animal Development* (Ed. R. WEBER; Academic Press, New York, London 1967), vol. 2, p. 193.
[23] M. FISCHBERG and A. W. BLACKLER, *Biological Organization* (Academic Press, New York, London 1963), p. 111.
[24] L. IFFY, Repr. Med. *5*, 96 (1970).

Embryologic and clinical indications of teratogenic action of delayed ovulation and/or insemination in man have often been reproted. HERTIG[10] finds increased pathology of cleaving stages and blastocysts derived from eggs inseminated later than the 14th day of the menstrual cycle. IFFY[24] has collected a large material on pregnancies which started in the last week of the menstrual cycle. He holds that delayed ovulation and/or delayed fertilization are major causes of reproductive pathology and on this basis propounds his 'past-mid-cycle theory', contra-indicating the practice of the rhythm method in birth control.

The realization of essentially good prospects to learn about the gene content of chromosomes from correlated studies of aneuploid karyotypes and malformations cannot be hoped for without paying due consideration to modified developmental conditions created by overripeness. This will not detract from the value of parallel immunologic and cytologic analysis with refined new technics[17, 25].

E. WITSCHI

The Population Council, Bio-Medical Division, The Rockefeller University, York Avenue and 66th Street, New York (N.Y. 10021, USA), 31 April 1971.

[25] M. A. FERGUSON-SMITH, *Birth Defects* (Orig. Articles Series, New York 1969), vol. 5, p. 3.

PRO EXPERIMENTIS

A Spectrophotometric Method for the Direct Recording of Dioldehydrase Activity

Dioldehydrase is a widely distributed enzyme among micro-organisms. The reaction which it catalyzes involves the dehydration of a number of diols[1] with production of an aldehyde, as indicated in the following general equation:

$$R\text{–}CHOH\text{–}CH_2OH \rightarrow R\text{–}CH_2\text{–}CHO + H_2O$$

The most commonly used substrate is propane-1,2-diol and the rate of the reaction is usually followed by the amount of 2,4-dinitrophenylhydrazone formed at a given time upon addition of 2,4-dinitrophenylhydrazine. This method, described by BOEHME and WINKLER[2], does not allow direct and continuous measurements of kinetics and, like most sampling methods, is rather time-consuming.

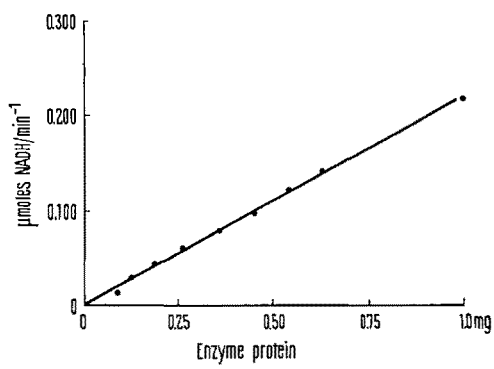
Since one of the products of the reaction is an aliphatic aldehyde we have attempted to couple the dioldehydrase reaction to that catalyzed by alcohol dehydrogenase. The rate of oxidation of NADH by the latter enzyme may be followed spectrophotometrically. The technique developed for a continuous recording of dioldehydrase activity is described in this paper.

Materials and methods. As a source of the apodioldehydrase, a cell-free extract from *Aerobacter aerogenes* (ATCC 8724) was prepared according to LEE and ABELES[3]. At a variance from the procedure described by the authors quoted we have omitted propanediol from the extraction mixture without appreciable loss of activity. The enzyme extract could be stored lyophilized up to 15 days.

The enzyme activity was followed by the rate of disappearance of NADH, as measured with a recording Eppendorf photometer equipped with a 366 nm filter. The cuvette holder of the instrument was thermostated at 30 °C. The assay mixture (1 ml) consisted of: apodioldehydrase in the range of 0.5–0.6 mg protein; 3 nmoles dimethylbenzimidazolyl cobamide (DMBC) (Glaxo); 0.5 mg alcohol dehydrogenase from yeast (SIGMA) (200–250 U/mg prot.); 0.9 µmoles NADH (SIGMA, Grade III); 50 µmoles *Tris*-HCl; 12.5 µmoles KCl and 0.3 mg Bovine Serum Albumin (SIGMA). The reaction is started by adding 3 µl of 1,2-propanediol (Fluka) stock solution (1 *M*) standardized according to EIBL and LANDS[4]. The reference cuvette contained buffer and 0.3 µmoles NADH. The addition of NADH also to the reference cuvette allows to use a high enough NADH concentration in the sample cuvette.

Propionaldehyde (Fluka) used in *Km* experiments was purified by redistillation. The redistillated product displayed a refractive index $n_D^{19} = 1.3643$ in agreement with data reported in literature ($n_D^{19} = 1.3646$)[5]. The aldehyde concentration was determined by addition of sodium bisulfite and iodimetry. Styreneglykol solution, used in experiments of specific inhibition, was made of pure reagent supplied by Fluka.

Results. A pre-requisite for the use of ADH as coupling enzyme for the spectrophotometric determination of dioldehydrase is that the dehydrogenase may react with propionaldehyde at a sufficiently high velocity and with a reasonable *Km*. Accurate determination of this parameter has yielded a value of $3.36 \times 10^{-3} M$.



Linear relationship of NADH oxidation rate with protein concentration. Experimental conditions as described under materials and methods.

[1] H. P. C. HOGENKAMP, Ann. Rew. Biochem. *37*, 225 (1968).
[2] H. BOEHME and O. WINKLER, Z. analyt. Chem. *142*, 1 (1954).
[3] H. A. LEE JR. and R. H. ABELES, J. biol. Chem. *238*, 2367 (1963).
[4] H. EIBL and W. E. M. LANDS, Analyt. Biochem. *33*, 58 (1970).
[5] P. G. STECKER, *The Merck Index* (Merck and Co. Inc., Rahway, N.Y., USA 1968).