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].

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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.

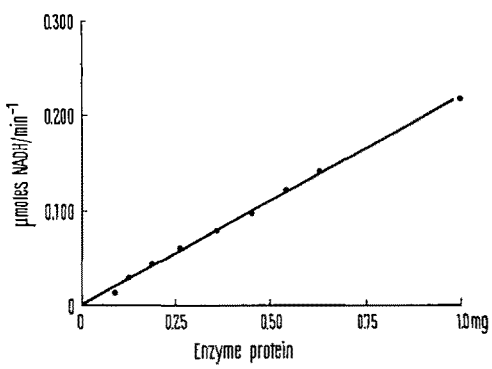
Since one of the products of the reaction is an aliphatic aldehyde we have attempted to couple the dioldehydrase reaction to that catalyzed by alcohol dehydrogenase. The rate of oxidation of NADH by the latter enzyme may be followed spectrophotometrically. The technique developed for a continuous recording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

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Since the ADH reaction should never be rate-limiting, relatively high concentrations of ADH must be added to the assay mixture in view of the low affinity of ADH for propionaldehyde. Preliminary experiments have shown that the enzyme activity becomes independent from ADH concentrations only above 50–100 µg of ADH per sample. A safe amount of enzyme, in the assay mixture, was considered to be 500 µg.

Another pre-requisite for the appropriate use of a coupled system to assay the dioldehydrase activity is that no other reaction involving NADH oxidation occurs in the system. The specificity of the assay has therefore been checked, as reported in Table I. It may be seen that unspecific interference in our preparations accounts for at most 7%. Most probably such an interference may be completely excluded by the use of respiratory inhibitors. Under the experimental conditions assessed, a strict proportionality was found between rate measured and amount of enzyme protein added to the assay mixture. This is clearly illustrated by the graph reported in Figure 1.

A direct comparison of the method described with the conventional dinitrophenylhydrazone method is reported in Table II. Clearly the results obtained by the 2 methods are perfectly comparable but the extent of the experimental error is definitely lower with the method we propose than with the method of BOEHME and WINKLER[2]. The standard error found in the former case is 6–7 times less than in the latter.

Some kinetics parameters of dioldehydrase from *A. aerogenes* have been also reinvestigated by this technique and compared with those reported in literature. Data reported in Table III indicate a good agreement of our data with those already published by other authors[6].

Discussion. The method described has the advantage of being a continuous recording of the kinetics rather than a sampling method. This allows accurate measurements of initial rates and, in addition, leaves much less room for experimental error. The sensitivity of the method is rather high as compared with the classical dinitrophenylhydrazone method. The sensitivity may be further increased by recording at 340 nm instead of 366 nm or even further by recording the fluorescence change of the nucleotide.

The method has limitations, however, being appliable only to relatively purified systems. This limitation may be, to a certain extent, circumvented by the use of the respiratory inhibitors. A second limitation arises from the necessity of using high concentrations of purified ADH which should never be rate-limiting. Preliminary experiments are therefore necessary to ensure that this condition is fulfilled. This point makes the method far more expensive than the previous one. All things considered, however, we feel that the balance between advantages and disadvantages of the new method favours it and disfavours the sampling method of BOEHEME and WINKLER.

Table I. Specificity of ADH-coupled dioldehydrase activity

	Enzymic activity (%)
Complete system	100
DMBC	7
ADH	7
1,2-Propanediol	7
KCl	66
BSA	90
Apodioldehydrase	0

Experimental conditions as described under materials and methods.

Table II. Dioldehydrase activity determined by ADH-coupled reaction and according to BOEHME and WINKLER[2]

nmoles propionaldehyde · mg^{-1} protein · min^{-1}	
ADH-coupled system	Dinitrophenylhydrazone system
120.3 ± 0.91 (6)	119.6 ± 6.5 (6)

Data ± S.E.M. In parenthesis number of determination.

Table III. Comparison of some kinetics parameters of dioldehydrase from *A. aerogenes* determined by the new assay method with those reported in literature

	New technique	Literature
K*m* of 1,2-propanediol	$1.20 \times 10^{-4} M$	$0.81 \times 10^{-4} M$ (ref.[6])
K*i* of styrene glykol	$3.56 \times 10^{-2} M$	$3.84 \times 10^{-2} M$ (ref.[6])

Zusammenfassung. Es wird eine raschere und empfindlichere Methode zur Bestimmung der Aktivität von Dioldehydrase beschrieben.

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CONGRESSUS

Italy
5th Meeting of the International Study Group for Steroid Hormones

in Roma, 6–8 December 1971.

Biochemical and Clinical Aspects of Steroid Enzymology. Biological Aspects: a) Enzymes in steroid biosynthesis. b) Enzymes in steroid metabolism. c) Enzymatic histochemistry. Clinical Aspects: a) In vitro studies. b) In vivo studies. c) Diagnostic methods.

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