Acid Hydrolysis and UV-Feulgen Staining of DNA-Aldehyde

The use of 5 *N* HCl at 12 °C for Feulgen hydrolysis was introduced by ITIKAWA and OGURA[1] and subsequently followed by JORDANOV[2], DECOSSE and AIELLO[3] and FAND[4]. All these workers used the conventional Feulgen procedure for staining DNA-aldehyde. The introduction of a new UV-Feulgen technique by the author[5–7] shows promise in studies of this kind, since with this method a much greater number of DNA-aldehyde molecules take part in the reaction than is possible with the conventional Feulgen staining. Moreover, with this method dye-DNA reaction mechanism is also accelerated so that staining time is considerably reduced. This communication furnishes an account of the effect of hydrolysis in 6 *N* HCl at 20 °C, and in 12 *N* HCl at 20 and 35 °C, for varying periods of time, followed by staining with Schiff reagent under exposure to UV-light obtained from a Hanovia mercury arc lamp.

Schiff reagent used in this investigation was prepared according to De TOMASI[8] with basic fuchsin of BDH. The materials used were liver and intestine obtained from a Holtzman strain of rat, and from the lip of the golden hamster, *Mesocricetus auratus* that were fixed in 10% buffered neutral formalin. Paraffin sections (12 μm) were used throughout. For hydrolysis, 2 different strengths of hydrochloric acid were used, 6 *N* and 12 *N*, and the temperature of hydrolysis was 20 °C. Hydrolysis was also performed in 12 *N* HCl at 35 °C. For staining, sections were hydrolyzed in 6 *N* HCl for 1 min to 7 h, in 12 *N* HCl for 1 min to 2 h, and in 12 *N* HCl at 35 °C for 1 to 40 min, rinsed in water, stained with Schiff reagent for 7 min under exposure to UV-light obtained from a Hanovia mercury arc lamp at room temperature[5–7], rinsed in water ,dehydrated through a graduated series of ethanol, cleared in xylol and mounted in DPX.

Results of staining, as judged by visual examination of sections under the microscope, are tabulated in the Table, where 1+, 2+ etc. indicate progressive increase in staining with the optimum intensity designated as 5+.

From the Table it is evident that there is a progressive increase in the intensity of UV-Feulgen staining in materials hydrolyzed in 6 *N* HCl at 20 °C for 1 to 20 min of hydrolysis, thereafter the intensity of staining remained the same, forming a plateau up to 120 min of hydrolysis. The intensity of staining afterwards was slightly less but remained so until up to 420 min of hydrolysis. The observations on materials hydrolyzed in 12 *N* HCl at 20 °C also simulate the above, except that a marked increase in staining intensity occurred after 1 min of hydrolysis as compared with hydrolysis in 6 *N* HCl for the same length of time. In the case of hydrolysis in 12 *N* HCl at 20 °C, optimum staining was achieved after hydrolysis for 5 min and the intensity of staining remained the same up to 40 min of hydrolysis, thereafter it declined rapidly and finally after 120 min became totally negative.

When tissue sections were hydrolyzed in 12 *N* HCl at 35 °C, almost perfect staining resulted following hydrolysis for 1 min, and optimum staining was attained after 3 and 5 min of hydrolysis. Thereafter intensity of staining diminished progressively and after 40 min of hydrolysis total lack of staining resulted.

DECOSSE and AIELLO[3] in their study with alcohol-formalin-acetic acid fixed thick smears of human peripheral blood compared the effect of Feulgen hydrolysis in 1 *N* HCl at 60 °C with 5 *N* HCl at 26 °C for varying periods of time, followed by staining with the conventional Feulgen procedure; they noted a rather close similarity in the ascending slopes of the 2 methods. They further noted that optimum hydrolysis time with 1 *N* HCl at 60 °C is 14 to 16 min, thereafter the amount of DNA-Feulgen starts diminishing until, after hydrolysis for 60 min, the values drop abruptly. Their data with 5 *N* HCl at 26 °C, on the other hand, definitely show a peak after hydrolysis for 40 min, followed by a somewhat lower plateau upto 120 min of hydrolysis. Afterwards, the amount of DNA-Feulgen diminishes progressively if hydrolysis is continued for 19 h. FAND's[4] study with human anterior pituitary glands is also similar to that of DECOSSE and AIELLO, particularly in respect to hydrolysis with 5 *N* HCl at different temperatures. This author[4] has also shown a very close similarity between hydrolysis curves of tissue hydrolyzed in 5 *N* and 1 *N* HCl at 60 °C. Evidence is also presented here to indicate close similarity of the hydrolysis curve for 12 *N* HCl at 35 °C with curves for 5 *N* and 1 *N* HCl at 60 °C. The present study with liver, intestine and lip, after hydrolysis in 6 *N* HCl at 20 °C followed by staining with UV-Feulgen technique, also indicate close similarity with that of DECOSSE and AIELLO and with that of FAND, except that degradation of DNA is very slow in all the materials studied here. This is so because, in the present investigation, an appreciable loss of staining does not occur even if hydrolysis is continued for 7 h. It is needless to mention here that the temperature and strength of the acid are different in the cases compared. But the studies of the present author with 12 *N* HCl at 20 °C, though are a fair approximation of those of DECOSSE and AIELLO employing 5 *N* HCl at 26 °C and FAND employing the same strength of

[1] O. ITIKAWA and Y. OGURA, Stain Tech. *29*, 9 (1954).
[2] J. JORDANOV, Acta histochem. *15*, 135 (1963).
[3] J. J. DECOSSE and N. AIELLO, J. Histochem. Cytochem. *14*, 601 (1966).
[4] S. B. FAND, *Introduction to Quantitative Cytochemistry* (Academic Press, New York, London 1970), vol. 2.
[5] M. K. DUTT, Nucleus *12*, 154 (1969).
[6] M. K. DUTT, *The Encyclopedia of Microscopy and Microtechnique* (Van Nostrand Reinhold Publishing Co., New York 1973).
[7] M. K. DUTT, Acta histochem, in press (1975).
[8] J. A. DE TOMASI, Stain Tech. *11*, 137 (1936).

Time of hydrolysis, strengths of the acid and temperature and UV-Feulgen intensity

Strengths of HCl and temperature	Time of hydrolysis (min)																	
	1	3	5	10	20	30	40	50	60	90	120	150	180	210	240	300	360	420
6 *N* (20 °C)	1+	2+	3+	4+	5+	5+	5+	5+	5+	5+	5+	4+	4+	4+	4+	4+	4+	4+
12 *N* (20 °C)	3+	4+	5+	5+	5+	5+	5+	4+	2+	2+	nil							
12 *N* (35 °C)	4+	5+	5+	4+	2+	1+	nil											

the acid at different temperatures, also show some similarity with data obtained after hydrolysis in 1 *N* HCl at 60 °C for varying periods of time. In fact the result in the present study with 12 *N* HCl at 20 °C is intermediate between that of 5 *N* HCl at 26 °C and 1 *N* HCl at 60 °C. Therefore, the present author feels that the process of breakdown of purine in materials hydrolyzed in 12 *N* HCl at 20 °C starts quickly, and even more quickly in 12 *N* HCl at 35 °C, just like after 1 *N* HCl at 60 °C; but whereas in the latter case further degradation of the DNA complex and loss of apurinic acid are brought about by heat, in the former case with 12 *N* HCl these are caused by the optimum normality of the acid and temperature.

Résumé. On a étudié l'effet des conditions d'hydrolyse de tissus ammaliens par l'acide chlorhydrique concentré sur la coloration de Feulgen en lumière UV.

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A Rapid and Simple Method for the Detection of Mycoplasma and Other Intracellular Contaminants

There are many published methods for the detection of mycoplasmas in cultured eukaryotic cells (for reviews see[1, 2] and references within), most of which are complex, time consuming and often inaccurate giving false-negative results. Recently a method has been published[3] that involves detection of cytoplasmic DNA-containing cell contaminants by staining their DNA with either Hoechst 33258 or 4′-6-diamidino-2-phenylindole and viewing by fluorescence microscopy. This method, as are several others, is based on the fact that mycoplasmas and some DNA animal viruses replicate in the cytoplasm. However, one drawback of this procedure is that cells must be grown on coverslips. In the method I describe, a small culture vessel (e.g. a 6 oz medical flat) routinely used for sub-culture is adequate for the assay. An answer can be obtained within 2 h, thus making it feasible to check for the presence of mycoplasma in sister cultures before each experiment, as suggested by Levine[2].

The method I describe is a simpler and more sensitive double-label modification of the one described by Schneider et al.[4] and makes use of the fact that mycoplasmas synthesize the enzyme pyrimidine phosphorylase[5] which both hydrolyses uridine (and thymidine) to uracil (and thymine), and can 'salvage' pyrimidines to nucleosides by the reverse reaction; this means that exogenous uracil can be incorporated into RNA in mycoplasma-infected cells but not in uninfected cells[5, 6] where this enzyme is in the main absent (but see ref.[2]).

Methods. A 50 times concentrated mixture of ^{3}H-uridine and ^{14}C-uracil was added to a rapidly growing, subconfluent bottle of cells to final levels of 1 μCi/ml and 0.1 μCi/ml respectively, without changing the medium. This bottle was allowed to incorporate label for 1 h, after which time the medium was poured off and the attached cells washed twice with a balanced salt solution (e.g. Earle's, Hank's, phosphate buffered saline). 5 ml of 0.1% sodium dodecyl sulphate (SDS; w/v) was then added and, after leaving for 5 min to allow complete lysis, the viscous solution was poured into 10 ml of 20% trichloroacetic acid (TCA; w/v) at 0 °C. A further 5 ml of 0.1% SDS was used to rinse the culture vessel, and this was also added to the 20% TCA. The mixture was left for 20 min at 0 °C before filtration onto a Whatman GF/A or GF/C filter; this filter was washed sequentially with 20 ml of ice-cold 10% TCA, 10 ml of ethanol-ether (1:1 by vol.) and 10 ml of ether. Finally the filter was dried in an oven at 100 °C for 5 min, added to a vial containing PPO-POPOP toluene scintillant and counted in a Packard liquid scintillation spectrometer; the settings were such that ^{3}H and ^{14}C could be differentiated e.g. ^{3}H: window 50–400, gain 90%; ^{14}C: window 350–1000, gain 10%. With these settings ^{3}H is counted at 47% relative efficiency of counts, and ^{14}C at 35%; spillover from ^{14}C channel into ^{3}H channel is 15% of the corrected ^{14}C cpm, while there is no spillover of ^{3}H cpm into the ^{14}C channel. Background values were obtained by stopping the incorporation at time zero (i.e. immediately after addition of label), and processing exactly as above.

A comparison of ^{3}H-uridine and ^{14}C-uracil incorporation in healthy, mycoplasma-infected, and kanamycin-treated infected mouse L cells

Condition of cells	Corrected TCA-insoluble radioactivity (cpm)	
	^{3}H-uridine	^{14}C-uracil
Healthy cells	81,540	7
Infected cells	3,050	5,285
Kanamycin (200 μg/ml) treatment for 7 days	7,488	1,522

Total cellular DNA was isolated essentially as described by Marmur[7].

Results and discussion. The importance of using radioactively-labelled nucleic acid precursor compounds in a wide variety of types of experiments with tissue culture cells over the last 20 years or so is obvious. If cell lines are contaminated with mycoplasma, then the host's metabolism is radically altered [1–6]. For example, mycoplasmas synthesize pyrimidine phosphorylase, an enzyme that hydrolyses pyrimidine nucleosides to ribose and the free base. An important consequence of this is that the use of thymidine or uridine either to label cellular nucleic acids, or in the case of thymidine, to synchronize cells, becomes impractical, since these coupounds are degraded by the mycoplasma contaminants. The presence of pyrimidine phosphorylase, however, can be exploited in a convenient assay for the presence of mycoplasma.

Schneider et al.[4] labelled separate cultures with either ^{3}H-uridine or ^{3}H-uracil, purified total cellular RNA from both, and determined their specific radioactivities. Thus the ratio of the specific radioactivities of the RNA labelled with ^{3}H-uridine to the RNA labelled with ^{3}H-uracil gave an indication of elevated levels of uridine phosphorylase and hence of mycoplasma contamination. This method has now both been simplified and made more sensitive by using uridine and uracil labelled with different radioisotopes in the same culture vessel, and also speeded up by labelling for much shorter periods. Using 2 radioisotopes obviates the necessity for determining the precise amounts of RNA present.