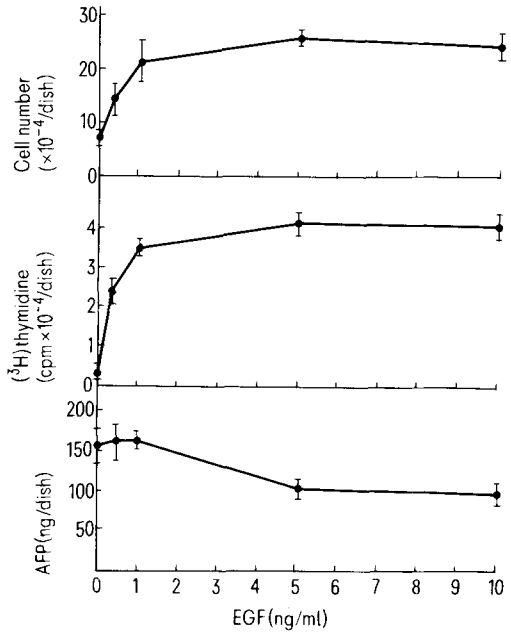
tion and stimulated AFP production of AH66 cells, antagonized EGF action on the hepatoma cells (table).

Discussion. EGF stimulated both [^{3}H]thymidine uptake and multiplication in AH66 cells, as reported for various other cells[3-5]. In the past EGF was reported to stimulate [^{3}H]thymidine incorporation without subsequent mitoses in the hepatocyte culture[10]. However, this dissociation seems to be due to the fact that the culture conditions and other factors necessory for normal hepatocyte division in vitro have not yet been fully defined[10]. Our results showed that the number of AH66 cells in cultures treated with EGF for 3 days was about 3 times greater than that of cells in cultures without EGF. If AFP production per cell is the same regardless of the presence of EGF, the medium after 3 days of culture of AH66 cells with EGF would be expected to contain at least 30 to 50% more AFP than the control culture. Our results showed that the amount of AFP produced by AH66 cells cultured with EGF was not significantly greater than that without EGF, suggesting the cells actively proliferating in the presence of EGF were synthesizing a smaller amount of AFP than the resting cells. However, we still do not know whether this is due to a direct effect of EGF on AFP production or secretion[11] or to a secondary effect resulting from the stimulation of cell proliferation by EGF[7,9].



Effects of EGF on the proliferation, [^{3}H]thymidine uptake and AFP production of AH66 cells. 5×10^4 of AH66 cells were cultured for 3 days in the low serum medium supplemented with various amounts of EGF. Cell number, [^{3}H]thymidine uptake and AFP concentration of the culture media were determined as described in materials and methods. Each point is the mean ± SD of triplicates.

The action of EGF on the proliferation of AH66 cells appeared to be similar to that of insulin (table). In the past it was shown that the receptors for EGF were distinct from the cell receptor for insulin and that EGF and insulin seemed to act additively[12]. However, since AH66 cells proliferating in response to EGF seem to be insensitive to insulin action (table), it seems not to be clear whether the 2 hormones act additively on hepatoma cells. EGF action on human fibroblasts is modulated by cholera toxin, theophyllin and Bt_2cAMP[13]. In AH66 cells, as well, the stimulation of cell proliferation by EGF was completely inhibited by Bt_2cAMP (table). Further details of the mechanism of EGF action remain to be studied.

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Elimination and metabolism of dimethylnitrosamine (DMN) by *Xenopus laevis* and other amphibians[1]

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Summary. The elimination of (^{14}C)-DMN after i.p. injection into *Xenopus* was measured, as was the metabolism in vitro of (^{14}C)-DMN by liver from *Xenopus* and 9 other amphibian species. In view of its rapid elimination from the body and low rate of metabolism by *Xenopus* liver in vitro, DMN is unlikely to be toxic or carcinogenic in *Xenopus*.

Nitrosamines have been shown to be toxic and carcinogenic in a wide range of vertebrate species ranging from fish[4, 5] to primates[6, 7]. The nitrosamines are not themselves active, but they can be dealkylated in some tissues; the resulting metabolite can alkylate cellular components including nucleic acids and proteins[8]. Species and tissue susceptibility to damage and tumour formation are to a certain extent correlated with the ability to metabolize nitrosamines.

Montesano et al.[9] found that the rate of metabolism of dimethylnitrosamine (DMN) by *Triturus helveticus* liver slices in vitro was comparable with that of rat kidney slices, and concluded that DMN would be expected to have toxic and/or carcinogenic effects on the liver of that species. However, they also found that injected DMN was very rapidly eliminated from the newt body into the surrounding water. Ingram[10] found that a single DMN injection had

Table 1. Recovery of (^{14}C)-DMN from groups of 10 *Xenopus* toadlets placed in 20, 50 or 100 ml water immediately after injection

Water volume	Time after injection (min)								
	5	10	20	30	45	60	75	90	120
	cpm recovered in water (% of total cpm injected)								
100 ml	24	42	68	78	86	90	92	93	93
50 ml	15	22	48	62	77	84	90	91	93
20 ml	10	18	35	46	55	58	64	65	67

Table 2. Metabolism of (^{14}C)-DMN by amphibian liver in vitro

Order	Species	No. of animals	Total No. of flasks	$^{14}CO_2$ production* (mean ± SE)
Anura	*Rana temporaria* (European common frog)	6	23	0.32 ± 0.05
	Bufo bufo bufo (common toad)	4	12	0.23 ± 0.04
	Rana esculenta (edible frog)	4	15	0.10 ± 0.02
	Xenopus laevis laevis (South African clawed toad)	6	23	0.07 ± 0.01
Urodela	*Notophthalmus viridescens* (American red-spotted newt)	4	6	0.22 ± 0.02
	Triturus alpestris (alpine newt)	4	7	0.19 ± 0.01
	Triturus vulgaris (common newt)	5	7	0.18 ± 0.01
	Amphiuma means (Congo eel)	1	4	0.15 ± 0.01
	Cynops pyrrhogaster (Japanese newt)	5	11	0.13 ± 0.03
	Triturus cristatus carnifex (Italian great-crested newt)	4	15	0.11 ± 0.01

* Percent ^{14}C added/160 mg liver/90 min.

Table 3. Effects of incubation atmosphere (air or 95% O_2/5% CO_2) on DMN metabolism by amphibian and rat tissues in vitro at 25 and 37 °C, respectively

Species/tissue	Atmosphere	No. of animals	No. of flasks	$^{14}CO_2$ production* (mean ± SE)	O_2 consumption (µl/h/g tissue)
Rat liver	Air	1	3	1.40 ± 0.19	983
	Oxygen	1	4	6.11 ± 0.93	1953
Rat kidney	Air	1	2	0.11 ± 0.01	1730
	Oxygen	1	2	0.19 ± 0.01	3417
Xenopus liver	Air	2	4	0.05 ± 0.01	209
	Oxygen	2	4	0.06 ± 0.01	264
Amphiuma liver	Air	1	4	0.09 ± 0.01	87
	Oxygen	1	4	0.10 ± 0.02	80
Rana liver	Air	4	4	0.23 ± 0.02	576
	Oxygen	4	4	0.27 ± 0.02	643

* Percent ^{14}C added/160 mg liver/90 min.

no discernible effects on *Triturus helveticus*, but 6 or 7 injections of DMN (16 g/kg) resulted in considerable liver damage, and tumours were found in 3 of 35 newts which survived for more than 2 months. Khudoley[11] induced tumours of the liver and haemopoietic tissue in *Rana temporaria* by dissolving 5 ppm DMN or 50 ppm diethylnitrosamine (DEN) in the tank water containing the frogs 6 times/week for periods of about 20 weeks.

For the past 5 years, we have been using DMN and DEN in attempts to induce tumours in *Xenopus laevis laevis* (the South African clawed toad). We have given immature toadlets and mature adults multiple injections of DMN and DEN equivalent to those given to newts by Ingram, and we have maintained tadpoles and toadlets in water to which DMN was regularly added according to Khudoley's procedure. Since none of these *Xenopus* have shown any sign of toxic damage or have yet developed tumours[12], we decided to study the rate of elimination of injected DMN from immature and adult toads, and to compare the capacity of *Xenopus* liver to metabolize DMN in vitro with that of liver from other anuran and urodele amphibians.

1. *Elimination of DMN from Xenopus.* 3 groups of 10 *Xenopus* toadlets, each weighing about 1 g, were anaesthetized in 1% MS222 (Sandoz, Basel, Switzerland), given i.p. injections of 0.1 mg (^{14}C)-DMN (5 mCi/mmole, Radiochemical Centre, Amersham, Bucks., England) in 0.05 ml water, then placed in 20 ml, 50 ml or 100 ml water to recover. The tank water was sampled at regular intervals for the next 2 h and its radioactivity measured by scintillation counting. Within 60 min, virtually all the injected radioactivity had been eliminated from toadlets recovering in 50 or 100 ml, while about 58% had been eliminated from the toadlets in 20 ml (table 1).

In a further experiment, large adult female *Xenopus*, each weighing about 85 g, were anaesthetized, injected with 5 mg (^{14}C)-DMN in 0.5 ml, then placed in small volumes of water (50 ml) to recover. 25% of the injected radioactivity had been eliminated into the water 1 h later, and 35% was recovered after 3 h. The amount of radioactivity in the water did not increase during the next 40 h, but when the volume of water around each animal was increased to 500 ml, radioactivity equivalent to a further 50% of that injected appeared in the water within 2 h.

Thus, in order to maximize exposure of body tissues to injected chemicals, it is necessary to keep *Xenopus* in the minimum volume of water compatible with their recovery from the anaesthetic and survival.

2. *Metabolism of DMN by amphibian liver in vitro.* The rates of metabolism of DMN by liver fragments from adults of 4 anuran and 6 urodele amphibian species were compared by a method based on that of Montesano et al.[9]. Livers were cut with fine scissors into 0.5 mm cubes, weighed and

transferred to Warburg flasks containing 2 ml of minimum essential medium-based amphibian organ culture medium[13]. (^{14}C)-DMN was added to each flask to give a final concentration of 60 µg and 0.1 µCi. The flasks were then shaken at 25 °C for 90 min in an air atmosphere. Radioactive CO_2 was trapped by NaOH in the centre well of each flask and its radioactivity determined by scintillation counting after conversion to (^{14}C)-$BaCO_3$ according to the method of Swann[14].

The results (table 2) show a considerable variation in the ability of the livers of the amphibian species tested to metabolize DMN. Liver from *Rana temporaria,* the species used by Khudoley[11] produced significantly more radioactive CO_2 than that from any other species, whereas *Xenopus* liver metabolized less DMN than liver from any other species.

3. *The effects of oxygen on DMN metabolism in vitro.* Montesano et al.[9] used an oxygen atmosphere in their work on DMN metabolism by fish and amphibian liver slices, but in our previous work on amphibian liver organ culture we have always used a zwitterionic buffered medium in free gaseous exchange with the atmosphere[13]. In a further series of experiments we therefore compared DMN metabolism by liver and kidney from a 200 g adult male Wistar rat and liver from *Xenopus* and 2 other amphibian species in Warburg flasks either filled with air or gassed with a 95% O_2/5% CO_2 mixture at the beginning of the test period.The results (table 3) show that the use of an oxygen atmosphere significantly increased the ability of the rat tissues, particularly the liver, to metabolize DMN in vitro, but had no significant effects on radioactive CO_2 production by liver from the amphibians. In addition, there were no significant differences in oxygen consumption by the 2 types of amphibian preparation (measured by Warburg manometry[15]), but the rat tissues incubated in 95% O_2/5% CO_2 used twice as much oxygen as those in air.

These results show that *Xenopus* eliminate injected DMN very rapidly when placed in the relatively large volumes of water in which these fully aquatic amphibians are normally maintained. Although some injected DMN appears to be retained in the body when *Xenopus* are kept in very small volumes of water, the comparatively very low rate of DMN metabolism by *Xenopus* tissues in vitro suggests that insufficient active metabolite would be produced for alkylation of cellular components to occur at the levels associated with toxic damage and/or tumour induction in rats[16]. Differences in rates of metabolism of DMN provide an explanation for our failure to induce tumours in *Xenopus* using procedures which, according to Ingram[10] and Khudoley[11], resulted in tumour induction in *Triturus helveticus* and in *Rana temporaria.*

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Chromatin fluorescence by pyronin staining[1]

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Summary. Human, chicken and mouse cells from different tissues show a bright red-orange fluorescence of the chromatin after staining with pyronin Y. The possibility that intercalation of the dye into double helical nucleic acids accounts for this fluorescence pattern is briefly discussed.

Pyronin Y (G) is a basic dye of the xanthene group, which is of considerable importance for nucleic acid cytochemistry[2-5]. Although this stain seems to be only relatively specific for RNA – e.g. see Kasten[6] and Pearse[4] –, its use in combination with methyl green as well as in the Brachet test[7] have proved very suitable for analyzing the occurrence and distribution of RNA in light microscopy[4,8]. During the course of cytological studies on the staining properties of pyronin Y, we have obtained unexpected results which are the subject of the present communication.

Smears of human and chicken blood were fixed in methanol for 2 min and air dried. Metaphase cells from human leukocyte cultures, and meiotic cells and spermatozoa from mouse testes were prepared according to the air drying method as usual. Staining was performed at room temperature, by using solutions of pyronin Y (Gurr) in distilled water at different concentrations, ranging from 0.74×10^{-1} M, (2%) to 0.74×10^{-7} M. Staining time was 5 min, after which slides were briefly washed in distilled water and air dried. Observations and photography were carried out using a Zeiss Photomicroscope III equipped with an epi-fluorescence condenser III RS. Preparations were analyzed under oil immersion using ×40 and ×100 objectives and green excitation filters.

After staining with considerably diluted pyronin Y solutions (0.74×10^{-5} M), nuclei in all preparations show a bright red-orange fluorescence (figure). The cytoplasm of blood cells with high RNA content (lymphocytes, monocytes) shows a scarce pale orange fluorescence. Mounting of preparations in different media (water, immersion oil, or