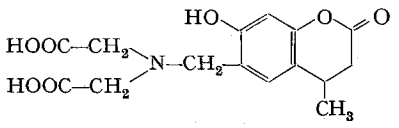
fuchsin stain. The use of haematoporphyrine is also limited, primarily because the high doses (300 mg/kg) necessary to obtain distinct labelling[12] may result in lethal intestinal complications. Furthermore, it has been observed that haematoporphyrine as well as alizarin red S is deposited at a somewhat different site from that of tetracycline and DCAF[13]. The advantages of multiple labelling and the above-mentioned difficulties prompted a search for other compounds for possible use as fluorescent labels.

Calcein-blue



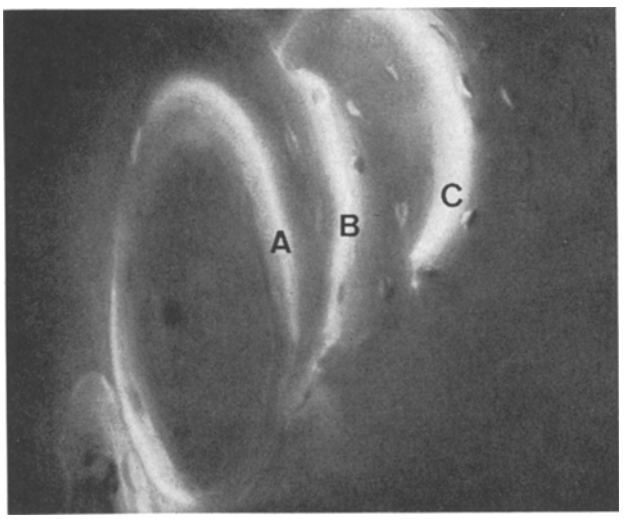
Calcein-blue[14], an indicator for the complexometric determination of Ca, Sr and Ba was tested. It was dissolved as a 3% solution in 2% $NaHCO_3$ and injected in sheep (i.v.), rabbits (i.v.) and rats (i.p.) at a dose of 30 mg/kg. The experimental animals were sacrificed from 24 h to 5 weeks after injection and the long bones fixed in alcohol. Sections 50 μ thick were prepared and examined microscopically under UV-illumination.

Calcein-blue was found to produce a blue fluorescence in bone upon UV-excitation[15]. The blue fluorescence appears at the same site as tetracycline and the fluoresceins. At a dose of 30 mg/kg no toxic effect was detected in the experimental animals and bone deposition was found to be unaffected. The fluorescence of Calcein-blue fades somewhat more quickly than the fluorescence of other labels, but sufficient time is provided to prepare photomicrographs. The contrasting fluorescence of Calcein-blue encourages its combination with a tetracycline[16] and a fluorescein[5,6] to obtain a trichrome UV-fluorescent labelling compatible with fuchsin counterstain[17].

Zusammenfassung. Calceinblau ergibt, in Knochen eingebaut, unter UV-Bestrahlung eine klar erkennbare blaue Fluoreszenz. Calceinblau eignet sich vor allem für Mehrfarbmarkierung des Knochens in Kombination mit Tetracyclin und Calcein oder DCAF. Solche Mehrfarbmarkierungen erleichtern das Studium des Knochenumbaus.

B. A. RAHN and S. M. PERREN

Laboratory for Experimental Surgery,
Swiss Research Institute,
CH-7270 Davos (Switzerland), 3 December 1969.



A contrasting, trichrome fluorescent label in a rabbit osteon (A, blue; B, green; C, yellow) is provided by combining Calcein-blue with the fluorescein DCAF and the tetracycline Achromycine®. The labels were injected at time intervals of 10 days.

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[14] Source: Fluka AG, CH-9470 Buchs.
[15] To observe the blue fluorescence a yellow barrier filter must be avoided.
[16] Achromycine® was kindly submitted – through the intermediary of their distributors Opopharma AG, Zürich – by Lederle Laboratories.
[17] We should thank Miss W. HUNTER for her skilful technical assistance.

Carcinogenic Action of Dimethylnitrosamine in Trout not Related to Methylation of Nucleic Acids and Protein in vivo

The carcinogenic action of dimethylnitrosamine has been correlated with the in vivo methylation of nucleic acids and proteins in the target organs[1]. Dimethylnitrosamine is not only a strong, mainly hepatocarcinogen in mammalia[2–5] but is also highly active in rainbow trout[6]. It is generally agreed that methylation is not due to the direct reaction of the unchanged molecule but to an intermediate formed by enzymatic oxidation[1,7,8], even if the formation of diazomethane in vivo is excluded[8].

Comparative studies by GAUDETTE et al.[9] have revealed that fish and certain type of amphibia lack the ability of oxidative drug metabolism, and we have now investigated whether 7-methylguanine is formed in the RNA of pigeon, frog and rainbow trout liver, species of different evolutionary stages, after application of ^{14}C-dimethylnitrosamine.

^{14}C-dimethylamine-hydrochloride was purchased from NEN, Chicago, and converted to the corresponding nitro-

Table I. Specific activity of RNA from liver in different species 6 h after application of ^{14}C-dimethylnitrosamine

Species	No. of animals	Route of application	Dosage of ^{14}C-DMNA/kg	Specific activity DPM/mg RNA
Pigeon	2	i.v.	25 µCi/0.84 mg	224
Frog	20	i.p.	25 µCi/0.36 mg	344
Trout	10	i.p.	25 µCi/0.36 mg	–

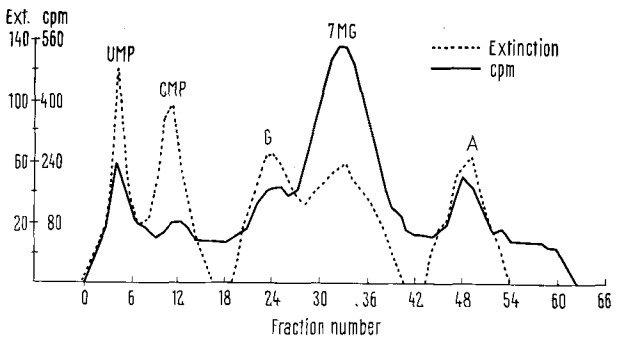

Fig. 1. Ion-exchange chromatography of an acid hydrolysate of RNA (50 mg) from pooled frog liver after treatment with 25 µCi/0.35 mg ^{14}C-dimethylnitrosamine. UMP, uridine-monophosphate; CMP, cytidine-monophosphate; G, guanine; 7-MG, 7-methylguanine; A, adenine. Carrier 7-methylguanine added.

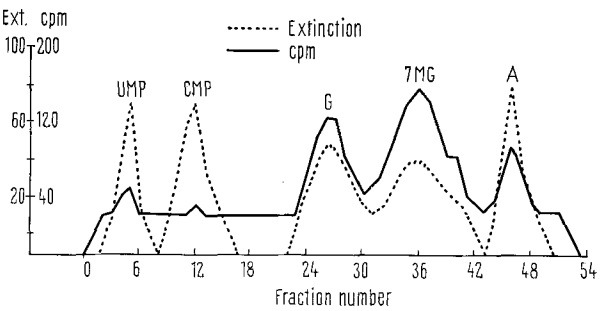

Fig. 2. Ion-exchange chromatography of an acid hydrolysate of RNA (30 mg) from pooled pigeon liver after treatment with 25 µCi/0.84 mg ^{14}C-dimethylnitrosamine. Carrier 7-methylguanine added; for explanation see Figure 1.

Table II. Specific activity of RNA, DNA and protein of trout liver after different routes of application and time of action of ^{14}C-dimethylnitrosamine in comparison to labelling of RNA after application of sodium formate

Substance	No. of animals	Route of application	Dosage per kg	Time of action (h)	Specific activity DPM/mg RNA	DNA	Protein
^{14}C-DMNA	5	i.p.	100 µCi/30 mg	6	–	–	–
^{14}C-DMNA	3	i.m.	100 µCi/30 mg	8	–	–	–
^{14}C-DMNA	4	i.m.	100 µCi/30 mg	15	–	–	–
^{14}C-formate	4	i.m.	300 µCi/3.6 mg	6	905	[a]	[a]

[a] Not determined.

samine as described by DUTTON and HEATH[10]. Sodium ^{14}C-formate was obtained from the Radiochemical Centre, Amersham. Commercial adult homing pigeons (300–400 g), rainbow trout (300–400 g), and frog (*Rana esculenta*, 40–45 g) were used. Frogs were kept in a moist cage and trout in an aquarium with flowing aerated water. Pigeons were injected into the brachial ala vene, frogs i.p. and rainbow trout i.p. or i.m. into the dorsal muscle. RNA and DNA from pooled livers were prepared by the modified procedure of KIDSON, KIRBY and RALPH[11,12]. After removal of RNA and DNA the protein-containing layer was poured into 5 vol. of methanol and the precipitate treated as described by MAGEE and FARBER[1] to purify the protein. RNA was hydrolyzed with 1 *N* HCl and chromatographed on a Dowex column (WX 50 200–400 mesh) by a 1–3 *N* HCl gradient. Radioactivity was determined with a Beckman SL 150 liquid scintillation counter.

It can be seen in Table I that the RNAs of pigeon and frog livers were labelled after treatment with ^{14}C-dimethylnitrosamine, whereas the RNA of trout liver showed no ^{14}C incorporation. The chromatographic elution patterns (Figures 1 and 2) showed that the RNA of both pigeon and frog liver contained 7-methylguanine. Table II shows that neither increased dosages of ^{14}C-dimethylnitrosamine, nor changes in injection site, nor different times of action lead to labelling of RNA, DNA and protein of trout liver. The RNA of this latter organ, however, was

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labelled when ^{14}C-formate was injected, confirming that the site of injection was correctly chosen for substances to reach the liver.

It must therefore be concluded that the carcinogenic and alkylating actions of dimethylnitrosamine are not correlated in trout, as has already been suggested for other nitroso compounds in rats[13–16].

Zusammenfassung. Zur Klärung des Zusammenhanges zwischen carcinogener und alkylierender Wirkung von Dimethylnitrosamin wurde die Bildung von ^{14}C-7-Methylguanin in der Leber-RNA von Tauben, Fröschen und Forellen nach Gabe von ^{14}C-Dimethylnitrosamin untersucht. Dabei konnte ^{14}C-7-Methylguanin in der Leber-RNS von Tauben und Fröschen nachgewiesen werden, während RNA, DNS und Protein der Leber bei Forellen nicht markiert wurden.

F. W. KRÜGER, G. WALKER and M. WIESSLER

Institut für experimentelle Toxikologie und Chemotherapie, Deutsches Krebsforschungszentrum, D-69 Heidelberg (Germany), 26 November 1969.

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A Novel Type of Granules Observed in Toad Endothelial Cells and Their Relationship with Blood Pressure Active Factors

We previously found[1,2] that the endothelium of the aorta, iliac and renal arteries of the toad is made of typical cells, the cytoplasm of which contains abundant granules visible with conventional electron microscopy techniques. Since we are searching for similar structures in different species of this and other classes, we are also interested in knowing whether these granules are linked to any special function or contain biologically active substances. Though the chemical agents contained in these granules are not known, we found that these bodies can be recovered apparently intact, together with contaminant mitochondria, in the pellets of subcellular fractions obtained by differential centrifugation. These fractions are rich in hypertensive activity which becomes rapidly unsedimentable when the pellets are suspended in a hypotonic medium.

The toad aorta aqueous extracts exhibit a strong hypertensive activity as compared with other tissues and organs of the same amphibian. The activity in kidney (Table) is also high and it is well known that this organ contains factors which act on blood pressure[3].

The hypertensive activity of the toad aorta becomes sedimentable to a great extent when the homogenates are prepared with isosmotic sucrose. The homogenates were prepared and fractionated as follow. Six local common toads *Bufo arenarum* H. were demedullated; the segments of the aorta between the junction of the aortic arches and iliac bifurcation were removed and placed in a small mortar with 1 ml of ice-cool 0.25 *M* sucrose in 0.01 *M tris*-HCl buffer (pH 7.4). They were minced with scissors and gently homogenized with the glass pestle; the suspension was filtered through a folded cheese-cloth. The volume of the filtrate was made up to 4 ml with the buffered sucrose and centrifuged in a refrigerated Beckman Spinco centrifuge with the No. 40.2 rotor. 4 sedimentable fractions and a final supernatant were recovered. Proteins were measured by LOWRY's method[4] and the hypertensive activity was tested in rats by the method of DE VITO et al.[5]

As is shown in Figure 1, fraction F1 exhibited high specific activity. The pellets activity was 93% extractable by osmotic shock in fractions N, F1 and F2, and 75%

Hypertensive activity in different toad tissues

	Aorta	Kidney	Brain	Muscle	Spleen	Liver
mm Hg/mg of tissue	13.7	13.3	2.9	2.1	0	0
mm Hg/mg of protein	210	185	52	45	0	0

From 40–60 mg of tissue were sonicated in 2 ml of 0.01 *M tris*-HCl buffer (pH 7.4) and centrifuged at 25,000 rpm for 10 min. Proteins and hypertensive activity were measured in the supernatants.

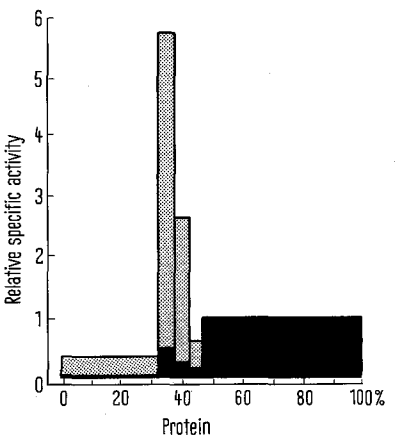

Fig. 1. Distribution of hypertensive activity in homogenates of *Bufo arenarum* H. aorta. The activity was measured in the fractions in mm of Hg/volume injected in the rat × volume of the fraction. Relative specific activity: percentage of total homogenate activity in the fraction/percentage of total homogenate protein in the same fraction. From left to right: the bars represent fraction N (600 *g*/5 min), F1 (9,959 *g*/3 min), F2 (39,825 *g*/7 min), F3 and the final supernatant (101,952 *g*/30 min). The first 4 fractions were suspended in 0.01 *M tris*-HCl buffer (pH 7.4) and spun at 101,952 *g*/30 min. The activity was tested in both supernatants and pellets (resuspended in the buffer) in order to calculate the percentage of released activity by osmotic shock (dotted area).