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Fluorophores as visualization aides in agar growth media

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Summary. A survey of 26 fluorophores revealed 5 which were non-inhibitory to *Staphylococcus aureus* and *Escherichia coli* and produced low background and high colony fluorescence.

Use of optical brighteners for staining living microorganisms has been reviewed by Paton and Jones[1]. They reported that some microorganisms could be stained by growth on agar which contained diamino-stilbenedisulfonate or oxycyanine compounds. Weaver and Zibilske[2] examined growth of bacterial suspensions in the presence of similar compounds and concluded that protein adsorption of the brighteners was the primary staining mechanism.

Other types of fluorescent compounds, those whose fluorescence is highly dependent on the immediate environment, have been widely used to study the conformation of membranes and proteins[3]. 2 factors which are important in determining the quantum yield of fluorophores are the viscosity and polarity (hydrophobicity) of the microenvironment. The bacterial envelope is a region of hydrophobicity and increased viscosity due to the presence of lipids, proteins, polysaccharides, and other closely related compounds. In designing a system for fluorescent detection of bacteria, one must take these properties into account. Compounds which have a high quantum yield when associated with membranes and proteins and a low quantum yield in an aqueous environment, such as an agar medium, are desirable. One fluorophore, 8-anilino-1-naphthalenesulfonate (ANS), was used by Cramer and Phillips[4] to study colicin-induced changes in *Escherichia coli* membrane conformation. We have observed that ANS could be incorporated into agars resulting in growth of highly fluorescent but otherwise normal bacterial colonies. This facilitated detection of transparent colonies and of colonies on opaque media, such as chocolate agar[5]. Media incorporating ANS and specific biochemicals were found to be useful in distinguishing between gram-positive and gram-negative colonies and in identifying bacterial species[6].

This report is a survey of fluorophores as additives to growth media with the objective of defining the characteristics of useful additives.

The compounds listed in the table were incorporated at 80 µg/ml in Nutrient Agar (Difco, Detroit, MI). The media were autoclave sterilized and plates poured. Separate plates were streaked for isolated colonies with *Escherichia coli* or *Staphylococcus aureus* and incubated overnight at 37°C. Plates were examined using long-wavelength UV-light. Each fluorophore was evaluated in terms of background fluorescence and overall visibility (fluorescence) of the colonies versus the agar, as described in the table. The

Effectiveness of fluorophores in nutrient agar for fluorescent labeling of *Escherichia coli* or *Staphylococcus aureus* colonies

Fluorophore	Agar surface[i]	Colony versus background[j]
A) Effective fluorophores		
1 8-Anilino-1-naphthalenesulfonic acid, Na or Mg salt[a]	−	+ +
2 2-p-Toluidino-6-naphthalenesulfonic acid[a]	−	+ +
3 1-p-Toluidino-8-naphthalenesulfonic acid[a,h]	−	+ +
4 Acridine orange[a]	−	+ +
5 1-Pyrenebutyric acid[a]	+	+ +
B) Less effective fluorophores		
1 1-Naphthalenesulfonic acid, Na salt[a]	−	− −
2 2-Naphthalenesulfonic acid, Na salt[a]	−	−
3 1-Naphthol-2-sulfonic acid, K salt[a]	−	− −
4 1-Naphthol-4-sulfonic acid, Na salt[a]	+ +	− −
5 2-Naphthol-6-sulfonic acid, Na salt[a]	+	− −
6 1-Naphthol-8-sulfonic acid, Na salt[a]	−	−
7 2-Naphthol-8-sulfonic acid, K salt[a]	+ +	−
8 7-Anilino-1-naphthol-3-sulfonic acid[b]	+	−
9 N-ethyl-naphthyl-2-amino-6-sulfonic acid[e]	+ +	− −
10 N-octadecylnaphthyl-2-amino-6-sulfonic acid[e]	− −	−
11 1-Naphthalenesulfonyl chloride[a]	−	−
12 2-Naphthalenesulfonyl chloride[a,h]	−	− −
13 5-Dimethylamino-1-naphthalenesulfonyl chloride[a]	+ +	−
14 2-p-Toluidino-6-naphthalenesulfonyl chloride[a]	−	− −
15 Lissamine rhodamine B sulfonyl chloride[a]	−	−
16 4-Amino-1-naphthol hydrochloride[a,h]	−	− −
17 Cycloheptamylose-dansyl chloride[d]	+ +	− −
18 Dansyl ethylamine[e]	− −	−
19 Rose bengal[a,g]	−	− −
20 Auramine O[f]	−	−
21 Fluorescamine[c]	+	− −

[a] Eastman Kodak Co., Rochester, N.Y. [b] Aldrich Chemical Co., Milwaukee, Wisc. [c] Hoffmann-La Roche, Inc., Nutley, N.J. [d] Pierce Chemical Co., Rockford, Ill. [e] ICN Pharmaceuticals, Cleveland, Ohio. [f] Fisher Scientific Co., Pittsburg, Pa. [g] Growth of *Staphylococcus aureus* inhibited. [h] Fluorogenic agent incompletely soluble at 90°C. [i] Fluorescence of colony-free agar surface: − low, + high, + + very high, − − poor. [j] Fluorescence of colony versus agar surface: − − poor, − mediocre, + good, + + excellent.

fluorophores in section A were effective as visualization aides. The compounds in section B were not effective agents, primarily because colonies could not be distinguished from the background due to high agar fluorescence or lack of colony fluorescence.

Both ANS and 2-p-toluidino-6-naphthalenesulfonic acid are practically non-fluorescent in aqueous solutions but in association with proteins or in non-polar (hydrophobic) environments, very large increases in the quantum yields occur (quantum efficiencies, non-polar/polar of 245 and 712, respectively[7,8]).

Acridine orange is another compound which works well in our system. The reason for its efficacy seems to be the frequently observed 'blue shift'[9] in its fluorescence emission maximum from orange in an aqueous environment to yellow in a more hydrophobic environment, i.e. in the bacterial envelope. This compound is known to affect the expression of plasmids[10], although we observed no effect on cultural characteristics. Pyrenebutyrate is useful due to another interesting phenomenon. The fluorescence of this compound is strongly quenched by dissolved oxygen. Vaughn and Weber[11] found, however, that if pyrenebutyrate were conjugated or adsorbed to bovine serum albumin, then fluorescence was unaffected by the presence of oxygen. This may be the mechanism by which fluorescent colonies occur on this agar.

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Effect of splenectomy on the humoral immune response in the lizard, *Scincus scincus*[1]

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Summary. Adult splenectomy in the lizard, *Scincus scincus*, did not affect humoral immune response to rat erythrocytes until 30 days post-immunization, but severely depressed subsequent antibody production.

It is well-known that adult splenectomy depresses but does not abolish humoral immune response in amphibians[2-4], birds[5,6] and mammals[7-12]. In contrast, splenectomy completely abrogates antibody production in lizards, *Calotes versicolor*[13-15]. No other study concerned with effect of splenectomy on reptilian humoral reactivity is available. Such shortage incited us to investigate the effect of splenectomy on humoral response of the lizard, *S. scincus*. Immune system of *S. scincus* in the different seasons has been described[16], and it was shown that lymphoid complex and capacity for antibody production are fully developed in summer and autumn. Splenectomy experiments were, therefore, performed in autumn.

Materials and methods. Adult male and female *S. scincus* (Scincidae, oviparous, hibernating[17]) weighing 20–40 g, were collected from the desert margin. Lizards were kept in large terraria, with 30 cm deep sand in a sunny animal room where the temperature in autumn ranged from 20 to 27 °C. Live insects and water were given ad libitum.

A group of 70 lizards were anaesthetized with ether and trunk region sterilized with ethyl alcohol. A small incision was made to the left of the midline opposite to the stomach, which was turned aside and the spleen excised by cauterizing the splenic blood vessels. After complete removal of spleen, the wound area was sprayed with Diacilin powder (Misr Co. Pharm. Ind., Cairo) and then the incision was closed with autoclips (Clay Adams, Parsippany, New Jersey, USA). Sham splenectomy was performed on a group of 70 lizards, by following all surgical procedures depicted above, except removal of spleen. Completion of the operation was ascertained by autopsy at the end of the investigation.

1 week after surgery, surviving 50 splenectomized, 50 sham-operated, as well as 50 intact lizards, received each a single i.p. injection of 0.3 ml of 20% rat erythrocytes suspension. 3–5 lizards from each group were sacrificed at a 15-day interval, over a period of 2 months and serum haemagglutinins assayed as described previously[16]. Significant differences between mean peak titers of the various groups were determined by the F-test (T Programmable 58, Texas Instruments, USA).

Results. Splenectomy experiments were performed in autumn 1976 and repeated in autumn 1977. Identical results were obtained and therefore pooled (figure). At 15, as well as, at 30 days post-immunization, splenectomized *Scincus* produced antibody levels close to those produced by sham-operated and intact lizards. There was no significant difference in antibody titers between the 3 groups. Serum antibody titers of intact and sham-splenectomized lizards rose rapidly, peak activity occurred at day 45, with a mean titer ($\log_2$) ranging from 12.1 to 13.8. As for the splenectomized lizards, serum antibody level began to wane and was significantly lower than antibody levels of intact and sham-operated *Scincus*, $p < 0.002$. On day 60, antibody titers of intact, sham-operated and those of splenectomized animals were still significantly different, $p < 0.005$.

Discussion. The present study indicates that in the lizard, *S. scincus*, adult splenectomy did not affect antibody production against rat red blood cells up till 30 days post-immunization. Serum haemagglutinin titer did not peak, however, and remained at a significantly lower level than in intact or sham-splenectomized lizards. Similar results were recorded in amphibians, birds and mammals, since adult splenectomy only depresses titer of circulating anti-