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Liquid Scintillation Counting Techniques for the Radioassay of [^{14}C] Melanin

Alan T. BULL

Department of Microbiology, Queen Elizabeth College (University of London), London, W. 8

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SUMMARY

The radioactive melanin used in this study was extracted from the cell walls of the fungus Aspergillus nidulans *after growth in the presence of ^{14}C-labelled precursors. The counting systems investigated comprised solid samples suspended in a liquid scintillator with or without silica gel, solubilized samples blended in a liquid scintillator and solubilized samples adsorbed on to glass-fibre discs wich were suspended in a liquid scintillator. Results obtained by counting solid samples with an end window detector are compared with the liquid scintillation data. High counting efficiencies were realized with the glass-fibre disc method which is recommended for the assay of soluble samples. Liquid scintillation counting of silica gel suspensions was vastly superior to end window counting for the assay of solid samples. The data suggest that ^{14}C-labelled materials of a particulate or highly pigmented nature can be counted with high efficiency in a liquid scintillation system.*

INTRODUCTION.

Work in this laboratory of the nature of microbial melanins has necessitated the development of a reliable counting method for these ^{14}C-labelled pigments. [^{14}C]-Melanin preparations have been counted using windowless and thin window gas-flow counters (Kim and Tchen, 1962; Chen and Chavin, 1965) and in liquid scintillation spectrometers (Mencher and Heim, 1962) but critical details of techniques are largely absent from the literature. Melanins are extremely intractable polymers and the following points should be considered when attempting to assay ^{14}C-labelled samples.

(i) solubility and chemical quenching : melanins are insoluble in most commonly used solvents and for a liquid scintillation counting procedure

they must be solubilized in aqueous alkali. Consequently the counting efficiency becomes much reduced due to the aqueous nature of the sample and further by the necessity for a blending agent such as ethanol to produce a one-phase system.

(ii) pigmentation and colour quenching : melanins are highly pigmented materials and absorb strongly over a wide range of visible and ultraviolet wavelengths. Ross and Yerick (1963) examined the problem of colour quenching in liquid scintillation systems and, using a water soluble coal-tar dye (E_{max} = 400 mμ) having rather similar absorption characteristics to *Aspergillus nidulans* melanin (Bull, 1966), showed that quenching was linear over a wide range of concentrations. Significantly the fluorescence peak of the commonly used PPO + DM-POPOP scintillator is about 420 mμ and that of BBOT about 430 mμ. In all experiments involving solutions of melanin, therefore, the pigment concentration has been kept below 0.05 absorbance units, at which level the colour quenching probably is less than seven per cent.

(iii) choice of counting method : a liquid scintillation method by virtue of its high sensitivity enables very small samples to be assayed. Chen and Chavin (1965) devised a method of counting melanin/protein mixtures on membrane filters with a Geiger-Mueller detector and, considering the size of their samples (0.14-0.65 mg), assumed the formation of infinitely thin films. We have obtained results of poor reproducibility when using this approach for counting melanin samples extracted from fungal mycelia. When sufficient [^{14}C]-melanin was available, films of infinite thickness have been prepared and the construction of a self-absorption correction curve used to extrapolate "true" specific activities (Wang and Willis, 1965). However, samples of more than 50 mg were required for the latter technique (see below), thereby precluding its routine use.

The choice of a liquid scintillation assay in the present context appeared to be the most advantageous and the systems investigated included (*a*) samples in solution in a scintillator liquid; (*b*) solid samples suspended in a scintillator liquid; (*c*) solid samples dispersed in a scintillator gel; and (*d*) samples in solutions adsorbed on to glass-fibre discs.

MATERIALS AND METHODS.

Counting apparatus. Samples were counted in a Model 3314 Packard Tri-Carb liquid scintillation spectrometer at —5°C; equilibration of samples at this temperature was made prior to counting. Discriminator settings on the red, green and blue channels were 50/1 000; 50/200; and 50/1 000 and the respecttive gain settings were 50, 8 and 8 per cent. Screwcap low-potassium glass vials (20 ml) were used to hold samples. In some experiments samples were adsorbed on to 2.1 cm diameter glass-fibre discs (Whatman GF/A), glass-fibre strips (Whatman GF 81), or dispersed in a fine grade silica gel (Cab-O-Sil

M-5, Packard Instrument Co., La Grange, Ill., U. S. A.). Counting was for a period of 100 minutes or a total of 2×10^4 counts.

In one experiment samples were counted as films on flat type aluminium planchets (Brilhart Ltd., Doncaster, U.K.) using an end window Geiger-Mueller detector with Automatic Scaler N530F (Ekco Electronics Ltd., Southend-on Sea, Essex, U.K.). Counting was for a period of a 100 seconds or for a total of 1×10^4 counts.

Reagents. Hyamine hydroxide was purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh. All other reagents were of AnalaR grade. Two scintillators were used in parallel experiments (i) a mixture of 2,5-diphenyloxazole (PPO) 0.3 % (w/v) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (DM-POPOP) 0.03 % (w/v) (Packard Instrument Co.) in toluene; (ii) 2,5-bis-[5'-tert.-butyl-benzoxazolyl(2')]-thiophene (BBOT) 0.8 % (w/v) (Ciba (A.R.L.) Ltd., Duxford, Cambridge, U.K.) in toluene.

Radiochemicals. DL-3(3,4-Dihydroxyphenyl)-2-[^{14}C]-alanine and [U-^{14}C] L-tyrosine hydrochloride were purchased from the Radiochemical Centre, Amersham, U.K. [^{14}C]-Melanin was extracted from the cell walls of *Aspergillus nidulans* grown in submerged culture in a chemically defined medium supplemented with one or other of the two labelled amino acids above (Bull, 1966). The extraction procedure was based on methods developed by Nicolaus, Piattelli and Fattorusso (1964).

Preparation of melanin samples. Hyamine hydroxide was the first choice solvent for melanin because it would produce a single phase system with the scintillator. Unfortunately the melanin samples were only sparingly soluble in this base. A 202 mg % (w/v) preparation was solubilized to the extent of 2 % after prolonged incubation at 45^0 C producing an intense brown-black solution. Complete solubilization was achieved in aqueous potassium hydroxide (2N) at 45^0 C. The resulting solution was a very pale straw colour which probably reflects a reversion to the leuco form of the pigment. This latter phenomenon reduces colour quenching and allows higher concentrations of melanin to be counted. In most experiments the final concentration of melanin in caustic solution was 143 μg/ml.

[^{14}C]-Melanin samples to be counted as solids in suspension or by a Geiger-Mueller detector were ground in an agate mortar such that all particles passed through a 300 mesh sieve, i.e. particles $< 53\ \mu$ diameter.

Determination of counting efficiencies.

Counting efficiencies for [U-^{14}C]-L-tyrosine hydrochloride and [^{14}C]-melanin in homogeneous solutions in liquid scintillator were determined via the channels ratio method (Bruno and Christian, 1961) and occasionally checked via internal standards. The specific activity of the [^{14}C]-melanin ($= S_0$) obtained

in this way was used to calculate the counting efficiencies of the other liquid scintillation systems studied (efficiency = observed cpm per mg/S_0).

Results and discussion.

Radio assay of L-Tyrosine. When [U-^{14}C] L-tyrosine HCl (specific activity = 34.4 μc/mg) was counted as a 0.2 ml aqueous sample in 5 ml of the PPO + DM-POPOP mixture, blending with 2.5 ml ethanol gave a maximum efficiency of 78.3 % as determined by channels ratio on an ethanol quenching correction curve for carbon-14. Amounts of ethanol smaller than 2.5 ml did not produce a homogeneous system. Table 1 shows the efficiency of counting the standard tyrosine in the two scintillators and also the effect of different proportions of ethanol. Hall and Cocking (1965) found that 30 % ethanol or 2-ethoxyethanol was the minimum practicable proportion required to establish a one-phase system with a 0.1 ml aqueous sample in 4-5 ml scintillator. The present results are in accord with these data and the extent of increasing ethanol quenching is similarly in close agreement.

Table 1. Efficiency of Counting a Standard [^{14}C]-Tyrosine

Scintillator	Ethanol (ml)	Efficiency (%)
(i) PPO + DM-POPOP (5 ml)	1.5 [a]	52.1
	2.5	78.3
	5.0	74.2
(ii) BBOT (5 ml)	1.5 [a]	53.7
	2.5	81.9
	5.0	68.2

0.2 ml aqueous sample (3.4 μg); specific activity 34.4 μc/mg.
[a] Heterogeneous phase system.

Radio assay of soluble melanin samples. The minimum volume of ethanol necessary to produce a one-phase system with 5 ml scintillator containing a 0.2 ml caustic alkali sample was found to be 5 ml. This is double the amount required by an aqueous sample (Table 1). The specific activity and efficiency data are presented in Table 2(*a*).

Radio assay of solid melanin samples : suspensions.

Two counting schedules were followed based on the studies of Hayes, Rogers and Langham (1956). In the first method, 5 mg of finely ground [^{14}C]-

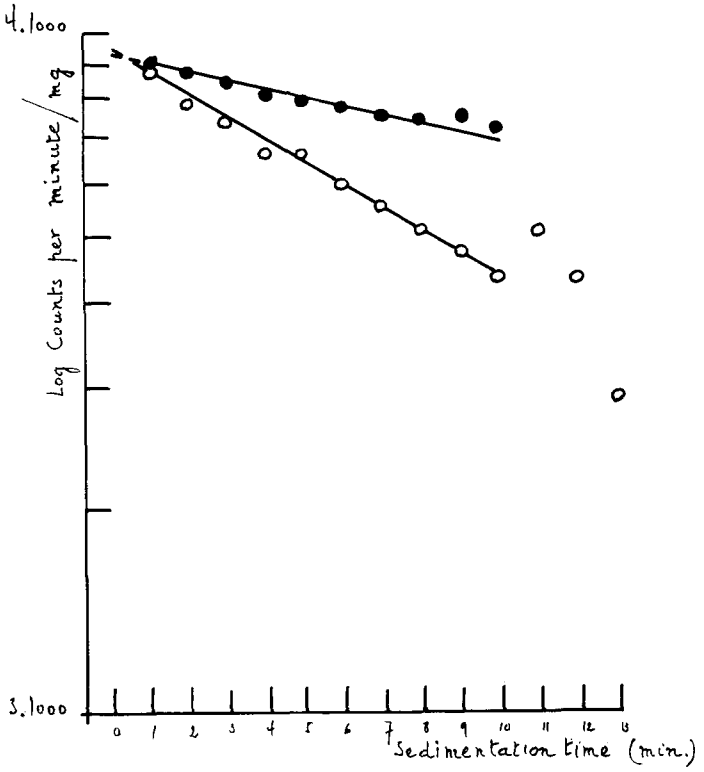
TABLE 2. Specific Activities and Counting Efficiency of [^{14}C]-Melanin Samples.

Sample	Scintillator	Ethanol (ml)	C.p.m./mg	E [a] (%)	Specific activity, S_0 (D.p.m./mg)
(*a*) 0.2 ml 2 N KOH solution (28.6 μg)	PPO + POPOP (5 ml)	5	1.647×10^4	67.0	2.458×10^4
	BBOT (5 ml)	5	1.514×10^4	61.6	
(*b*) Suspended solid in scintillator liquid (5 mg)					
Series A	BBOT (5 ml)	5	2.784×10^3	11.3	
Series B	BBOT (5 ml)	5	2.826×10^3	11.4	
Series C	BBOT (5 ml)	5	1.791×10^3	7.3	
(*c*) 0.1 ml adsorbed on glass-fibre disc (14.3 μg)	PPO + POPOP (0.2 ml)		1.828×10^4	74.3	
	PPO + POPOP (0.2 ml)	0.2	1.329×10^4	54.0	
	BBOT (0.2 ml)		2.132×10^4	86.7	
	BBOT (0.2 ml)	0.2	1.159×10^4	47.2	
(*d*) Suspended solid in 4 % silica gel (5 mg)	PPO + POPOP (10 ml)		1.048×10^4	42.6	
	BBOT (10 ml)		1.000×10^4	40.7	

[a] Efficiency (E) calculated as $\frac{\text{c.p.m. per mg}}{S_0}$, where S_0 is the specific activity of melanin counted in a homogeneous solution of the PPO + DM-POPOP scintillation mixture (E of this system was obtained from channels ratio).

melanin were added to a total of 10 ml scintillation mixture in a counting vial and equilibrated at —5° C. The vial was then hand shaken ten times and counted immediately for 60 seconds. This process was repeated ten times and the mean activity calculated (Table 2(*b*), series A); (standard error of mean = ± 5.28 %). Sedimentation of the suspension was accompanied by a decreasing count rate which Hayes *et al.* (1956) presume to parallel a drift away from 4π to 2π counting geometry as the sample settles on to the bottom of the vial. In the second method, therefore, a vial containing 5 mg labelled melanin in 10 ml scintillation mixture was shaken ten times by hand after temperature equilibration and counted immediately at 60 second intervals for 10 minutes. Over the initial 10 minutes there was an exponential fall in count rate which amounted to 518 cpm/mg; thereafter the count rate oscillated about a

FIG. 1. Liquid Scintillation counting of [^{14}C]-Melanin Suspensions : Effect of Sedimentation on Count Rate.



○ Initial sample (particle size < 53 μ diameter), .
● Ultrasonicated sample.

mean (Fig. 1). Extrapolation of the count rate curve to zero time gave a corrected value for the specific activity (Table 2(*b*), series B).

Major disadvantages of counting suspensions in liquid scintillators are those of light scattering and self absorption. Hayes and his associates found that a small particle size minimized self absorption and also stabilized the suspension. Accordingly, the [^{14}C]-melanin particles were further reduced in size by treating the suspension for 1 minute in a Kenny Pulsatron bath (Kenny's (Ultrasonics) Ltd., Basildon, U.K.) at an operating voltage of 850 v. The vial was re-cooled to —5^{0} C and after the standard shaking was counted again at 60 second intervals. Reducing the particle size induced greater suspension stability as predicted and over a 10 minute period the loss in count rate was only 237 cpm/mg. However, contrary to the results reported by Hayes *et al.*, the counting efficiency was reduced following ultrasonication (Fig. 1); Table 2(*b*), series C). Estimates of self absorption loss in count rate are given in Table 3.

TABLE 3. Self Absorption Losses in Suspension Counting Systems

Melanin preparation method	c.p.m./mg	f [a]	Percentage Self Absorption Loss = $(1 - f) \times 100$
Liquid suspension			
Series A	2.784×10^4	0.184	81.6
Series B	2.826×10^4	0.187	81.3
Series C	1.791×10^4	0.118	88.2
Silica gel suspension	1.000×10^4	0.664	33.6

[a] The ratio f is defined as $\frac{\text{Count rate in suspension}}{\text{Count rate in homogeneous solution}}$

RADIO ASSAY OF SOLID MELANIN SAMPLES : GELS.

Silica gels were prepared by first placing the Cab-O-Sil in the counting vial followed by the addition of the sample. Finally scintillator was poured into the vial which was shaken vigorously to form a clear gel in which the sample was dispersed as homogeneously as possible. A slight negative pressure applied to the vial removed trapped air bubbles. This procedure was recommended by the observations of Blanchard and Takahashi (1961). These authors reported that microgram amounts of material could be adsorbed on to the walls of the vial from the toluene solutions and thus produce non-linearity of counting rate when more activity was added to the vial. Consequently,

the order of addition of materials to the vials ensures that the activity is preferentially adsorbed on to the silica. Specific activity and efficiency data obtained with this method are given in Table 2(*d*). The gel system gave a much higher counting efficiency in comparison with liquid suspensions; up to a 4-fold increase when BBOT was the scintillator used. One factor here may be the stabilization of 4π geometry in the gel. Self adsorption loss data for the gel system are included in Table 3.

Radio assay of soluble melanin samples on glass-fibre supports. Recently Davis and Cocking (1966) have evaluated the use of glass-fibre discs in liquid scintillation counting. They found that ^{14}C-labelled protein in 0.25 N Na OH could be counted with an efficiency of 77 % in 1 ml of PPO (0.4 % w/v) + DM-POPOP (0.01 % w/v) in toluene. Application of samples to discs was by the means described by Davies and Cocking and drying of the discs was made with a 60 W lamp (Philips) mounted 6 cm above the discs. Drying to completeness was allowed to take place for 30-40 min (Davies and Cocking state that even traces of water seriously reduce the counting efficiency). Dried discs were placed horizontally in the bottom of counting vials and over layered with 0.2 ml scintillator liquid. Very high efficiency of counting of [^{14}C]-melanin resulted with this method, being 74.3 % for PPO + DM-POPOP and 86.7 % for BBOT. However, if an aliquot of ethanol was added to the scintillator, the efficiencies fell sharply to levels even lower than those obtained with solubilized samples in liquid systems blended with ethanol (cf. Table 2*c* and *a*).

The effect of altering the orientation of the glass-fibre support on counting efficiency also was investigated. Strips (16 × 25 mm) were cut from sheets of Whatman GF 81 glass-fibre (0.25 mm thick) and samples applied and dried. The strips were held vertically in counting vials and 10 ml BBOT scintillator added. [^{14}C]-Melanin was counted with an efficiency of 74.8 % (counting error = 1.9 %); this value is nearly 12 % less than similar counting on horizontally held discs. This is a somewhat surprising result as the vertical orientation might be expected to approach 4π geometry. However, Davies and Cocking (1966) have reported a 5-6 % fall in the efficiency of counting ^{14}C-labelled tomato protein on horizontal glass-fibre discs when they increased the volume of scintillator from 0.8 ml to 10 ml. Hence the present results may be a reflection of scintillator volume rather than orientation.

Backgrounds levels. Table 4 details the background counts obtained with the various liquid scintillation methods discussed above.

Radio assay of solid melanin samples : Geiger-Mueller counting. The relative specific activity of the [^{14}C]-melanin was determined by counting samples of increasing weight (constant specific activity) evenly distributed on a planchet with an end window Geiger-Mueller tube. The results are show in Table 5. From a logarithmic plot of « apparent » specific activity against sample weight, the "true" specific activity (sensu Willis and Wang, 1965, p. 204) was defined as 450 c.p.m./mg by extrapolating to zero sample thickness. The counting

efficiency based on data from infinitely thick films was 0.54 %, while a value of 1.83 % was obtained using the extrapolated value, i.e. data relating to infinitely thin films.

TABLE 4. Background Levels

Method	Volume of Scintillator (ml)	Ethanol (ml)	Additions	Background (c.p.m.) P [a]	Background (c.p.m.) B [b]
Liquid scintillator	5	5	Water 0.2 ml	72	73
Liquid scintillator	5	5	2 N KOH 0.2 ml	74	75
Liquid scintillator	5	5	Unlabelled melanin 5 mg	32	33
Glass-fibre dise	0.2		Water 0.1 ml	23	25
Glass-fibre disc	0.2		2 N KOH 0.1 ml	34	35
Cab-O-Sil gel	10		Water 0.2 ml	34	32
Cab-O-Sil gel	10		Unlabelled melanin 5 mg	34	30

[a] PPO + DM-POPOP.
[b] BBOT.

TABLE 5. Saturation Thickness (mg/3.8 cm^2) of a [^{14}C]-Melanin counted with an End Window Geiger-Mueller tube [a, b].

Sample (mg)	Counts/minute	Apparent Specific Activity (c.p.m./mg)
1.4	750	407
9.5	2 828	298
25.6	5 223	204
53.3	7 046	132
74.4	7 086	95

[a] Operating voltage 780 v.
[b] Background = 14 c.p.m.

CONCLUSIONS.

The results of this study recommend the use of glass-fibre discs and silica gels respectively for the radioassay of solubilized and solid specimens of [^{14}C]-melanin. The use of glass-fibre discs is attractive for several reasons —

high counting efficiency, economic amounts of scintillator required and the fact that little or none of the activity is transferred to the counting vial. BBOT has proved to be somewhat superior a scintillator to the POPOP + PPO mixture in the glass-fibre disc system. High efficiencies were obtained with BBOT and ethanol quenching only became a serious problem when the ratio of scintillator to ethanol was less than 2 to 1, Tables 1 and 2(*c*). In addition, the data of Tables 1 and 2(*c*) suggest that BBOT is less susceptible to colour quenching than PPO + DM-POPOP. BBOT has the added advantage in preparation of being a single component scintillator. Cab-O-Sil M-5 gels were used successfully to assay small solid samples of [^{14}C]-melanin and provided a satisfactory means of counting solid samples with a reasonably high efficiency. It is interesting to compare the disc and gel radioassay methods by calculating a figure of merit for each system; these data are presented in Table 6.

TABLE 6. Figure of Merit Values for Disc and Gel Counting Systems

System	Scintillator	Weight of melanin (mg)	Efficiency (%)	Figure of merit [a]
Glass-fibre disc	PPO + DM-POPOP	0.0143	74.3	1.063
	BBOT	0.0143	86.7	1.240
Cab-O-Sil gel	PPO + DM-POPOP	5.0000	42.6	213.0
	BBOT	5.0000	40.7	203.5

[a] Calculated as weight of melanin × efficiency.

The figure of merit values for the gel system are considerably greater than the corresponding values for the disc method, 200-times and 164-times greater with PPO + DM-POPOP and BBOT respectively.

However, the determination of counting efficiencies for suspension systems is very difficult whereas the determination of glass-fibre disc efficiencies can be made via the channels ratio method (Turner, 1967). Thus, when counting efficiencies for ethanol quenched glass-fibre discs were derived from channels ratios using a correction curve for homogeneous solutions, results within 9-11 % of the values quoted in Table 2(*c*) were obtained. For choice, therefore, the use of discs is preferred to that of gels. Finally, the data demonstrate the great superiority of silica gels over Geiger-Mueller counting for solid preparations.

The glass-fibre disc-BBOT system has been used to assay samples of [^{14}C]-melanin fractionated on Sephadex columns (Bull, 1966). Certain microbial melanins fade rapidly in caustic solutions in the presence of oxygen (B.I.

Rowley, personal communication); this is particularly true of extracellular melanins. Consequently, this sensitive radioassay has proved indispensable in the analysis of such material. The utility of the glass-fibre disc method has been warranted by the work of Davies and Cocking (1966) who have counted suspensions of tritiated plant protoplasts, and of Kelly (1967) who has used the technique to study the effect of organic substrates on an obligate chemoautotrophic bacterium. The present data encourage the use of liquid scintillation counting of highly coloured and particulate specimens of low specific activity and of specimens labelled with low energy β-emitting isotopes.

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