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ON THE QUANTITATIVE AMIDO BLACK B STAINING OF PROTEIN SPOTS IN AGAR GEL AT LOW LOCAL PROTEIN CONCENTRATIONS

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SUMMARY

Protein spots in agar gel of identical protein content but different in surface area are found to bind different amounts of dye upon staining with Amido Black B. The lower the protein concentration within the agar gel, the more the Amido Black B content of the spot falls short of the value expected from the amount of protein present, as measured by means of its ultraviolet-light absorption.

If, however, pure agarose is used instead of agar, the relationship between the protein and dye contents of spots is linear down to protein concentrations of 0.05 μg per mm^2 spot surface.

INTRODUCTION

During attempts to establish the ratio of the amounts of protein present in faint and strong spots in electrophoresis patterns stained with Amido Black, we found that it

seemed to depend on the concentration of the protein solution from which the electropherograms were made. The lower the concentration, the higher the ratio of the stain contents of the strong and the weak spots respectively.

Similar results were obtained if a series of droplets of identical volume but with decreasing concentrations of a protein were applied on an agar gel. In this case, too, the more faintly coloured droplets were found to contain less protein as calculated from the amount of dye present than would be expected from the known protein concentrations.

Finally, the amount of dye found in droplets that were identical in volume and protein concentration turned out to be dependent on the surface area of the spots, the larger spots containing less dye than the smaller ones.

The present paper describes experiments designed to, first, determine the amounts of protein present in the spots by a method independent of Amido Black staining, and, second, study the quantitative behaviour of the latter reaction.

EXPERIMENTAL

The apparatus used is essentially that described by WIEME[1] adapted for the use with agar electropherograms and protein spots not more than 4 mm wide. The pherogram (Fig. 1, S) is scanned by a succession of light spots provided by the holes in a rotating disk (D) illuminated from beneath. The aperture in the stationary screen (M) is 4 mm in length. The holes in the disk are also 4 mm apart, so that light falling through one hole at a time is admitted to the object. The slightly curved path of the holes is parallel to the electrophoresis lines. The pherogram is slowly transported in a direction perpendicular to the scanning line. Thus, all the features of the pherogram are successively scanned.

Light is provided by a quartz monochromator[2] illuminated by a high-pressure mercury arc (Philips HPW 125). The light falling through the scanning hole and the

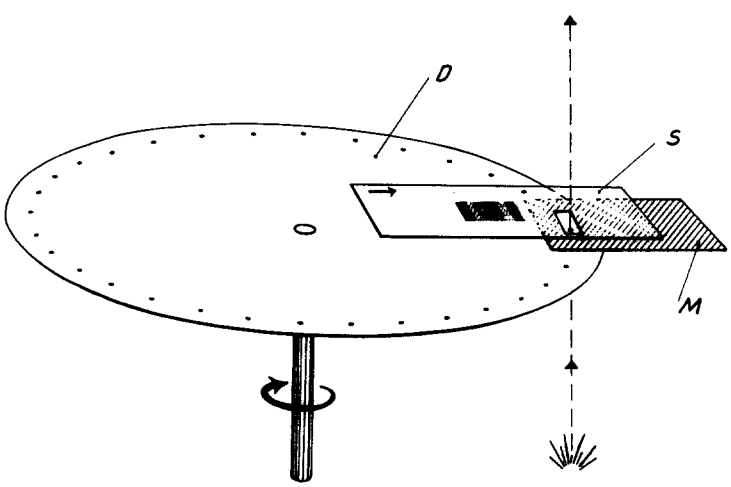

Fig. 1. Diagram of the essential optical arrangement. D, rotating opaque disk, carrying holes along its periphery; M, stationary mask, admitting light to the slide through one hole at a time; S, slide, slowly transported in the direction of the arrow.

pherogram is measured by a photomultiplier tube connected to a logarithmic amplifier after the design of TEN HAAF *et al.*[3]. The amplifier output is fed into a recorder that is too slow to follow the individual scanning pulses but fast enough to record a curve corresponding to the lines of the pherogram. As the recorder deflections are proportional to the integrated extinctions met by the light spots on their course across the electrophoresis pattern, the area under a recorded curve should represent a measure of the amount of light-absorbing substance present in the corresponding part of the slide. This area is measured by means of a planimeter.

On optically favourable objects, the measurements were found to be accurate to within a few per cent, but with agar slides the accuracy is occasionally less because of irregularities in its optical properties.

Specimens were prepared as follows: agar slides (either Difco agar or agarose prepared after ARAKI[4]) were made according to WIEME AND RABAEY[5], using quartz and glass slides. On the agar surface droplets of protein solutions were deposited by means of a micropipette containing approx. 0.5 μl. The same micropipette was used throughout. Some of these droplets were allowed to soak into the agar gel, thereby producing round spots approx. 3 mm in diameter, others were smeared out lengthwise resulting in oblong spots measuring approx. 3 $\times$ 10 mm.

Bovine albumin and horse γ-globulin were used in a concentration of approx. 1 % ("undiluted") and in a 3 -times diluted solution.

The slides were not subjected to electrophoresis but otherwise they were treated as electropherograms.

The quartz slides were measured prior to staining at 230 mμ, the shortest wavelength available in the present apparatus, At 230 mμ, the extinction of globulin and albumin is of the same order of magnitude as that after staining when measured at the absorption peak of Amido Black. Next, both the quartz and the glass slides were stained with Amido Black B and measured at 600 mμ.

RESULTS

The results of a typical experiment with globulin spots on agar (Difco) after staining with Amido Black B are given in Table I.

Table I bears out the facts mentioned in the INTRODUCTION. Notably, the results would suggest different protein contents in spots that differ from each other in their surface area only.

However, the measurements at 230 mμ of the quartz slides prior to staining reveals (Table II) that the protein contents of the round and oblong spots of the same concentration are equal.

Moreover, with the latter technique the dilution ratio of the two protein solutions is found to be 1:2.8 as compared to the values of between 1:4 and 1:6 to be derived from Table I.

These observations suggest the possibility of an interaction between *e.g.* sulfonic groups of the agar gel and the protein, resulting in a loss of sites available for the staining reaction. If the agar contains a restricted number of such groups their influence should be greatest at low local protein concentrations.

In order to verify this supposition, we performed a number of measurements on slides prepared exactly as for Table I, but with a sample of pure agarose prepared

TABLE I

INTEGRATED EXTINCTION VALUES AT 600 mμ OF HORSE γ-GLOBULIN SPOTS IN AGAR GEL (DIFCO) AFTER STAINING WITH AMIDO BLACK B

The unit is arbitrary. Equal drops of approx. 0.5 μl, protein concentration approx. 1 %, dilution ratio 1:3, spot sizes: round, 3 × 3 mm; oblong, 3 × 10 mm.

	Shape of spot undiluted		*Shape of spot diluted*	
	Round	*Oblong*	*Round*	*Oblong*
	160	148	50	30
	150	148	43	37
	180	114	50	28
	165	144	40	30
	166	112	31	28
	150	112	38	22
	174	124	41	30
	168	140	38	28
Mean	164	130	41	29
Standard deviation of mean	3.7	5.8	2.5	1.45

TABLE II

INTEGRATED EXTINCTION VALUES AT 230 mμ OF HORSE γ-GLOBULIN SPOTS IN AGAR AND AGAROSE GELS

The unit in this and the following table is the same as that in Table I.

	Shape of spot undiluted		*Shape of spot diluted*	
	Round	*Oblong*	*Round*	*Oblong*
Agar	132	125	51	49
Agar	129	132	43	42
Agarose	128	138	45	46
Agarose	130	130	46	46
Agarose	128	132	45	49
Mean	129	131	46	46
Standard deviation of mean	0.2	2.1	1.5	1.3

TABLE III

INTEGRATED EXTINCTION VALUES AT 600 mμ OF HORSE γ-GLOBULIN SPOTS IN AGAROSE GEL STAINED WITH AMIDO BLACK B

See Table I and II.

	Shape of spot undiluted		*Shape of spot diluted*	
	Round	*Oblong*	*Round*	*Oblong*
	201	209	76	71
	210	216	76	61
	209	205	69	69
	223	208	72	76
	210	223	74	76
	218	210	71	66
	209	204	68	56
	225	222	69	68
Mean	213	213	72	68
Standard deviation of mean	2.9	2.7	1.1	2.6

according to ARAKI. Table III summarizes the results obtained at 600 mμ after staining of the slides.

These results show that with this agarose the outcome of the measurements no longer depends on the local concentration of the protein. Accordingly, the dilution ratio as determined from the pooled measurements is reasonably close to the ratio found in ultraviolet light (1:3.0 instead of 1:2.8).

Entirely similar results have been obtained on albumin samples, though the ratio of the extinctions at 230 mμ (unstained) and 600 mμ (stained) is different from that found in globulin.

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