Changes in the visible absorption spectra of dyes due to binding with polyelectrolytes of high molecular weight are known as metachromasy and have been extensively studied. Recent advances in metachromasy have been summarized in several reviews (1–3). It is also known that metachromasy due to binding with proteins is generally weak but occurs with both acidic and basic dyes. Methyl orange (MO) and bromocresol green (BCG) as acidic dyes and toluidine blue and acridine orange as basic dyes have often been used in studies of metachromasy.

As pointed out by Klotz (4), the addition of a suitable organic acid to a solution of an MO- or azosulfathiazole-protein complex reversed the effect of protein on the spectrum. This reversal of metachromasy is considered to be due to the fact that the organic acid anion displaces the dye anion from the binding sites on the protein molecule. Thus it seems likely that the competitive ability of an organic acid against an acidic dye should be a measure of the strength of its binding with the protein molecule and in fact this has been confirmed by equilibrium dialysis binding studies.

In the present work the metachromasy of BCG with bovine serum albumin was chosen for study, since BCG was tightly bound to albumin and a relatively great spectral change was produced, as has been shown by Rodkey (5) and others (6). \( p\)-Aminosalicylic acid (PAS) is an important antituberculous agent but its activity is known to be greatly decreased in the presence of serum albumin. This indicates a high affinity of PAS for serum albumin. In this work the competitive ability of PAS was compared with those of benzoic acid (BA), salicylic acid (SA) and \( p\)-aminobenzoic acid (PABA) by spectrophotometry and information was obtained about the binding sites of PAS on the serum albumin molecule.

This information is suggestive for the structure of a suitable PAS derivative with antituberculous activity but less protein binding activity. Accordingly a new and more efficient antituberculous compound, aminobenzyl PAS is proposed.

**EXPERIMENTAL METHODS**

The BCG, BA, SA, PABA used in the present study were reagent grade compounds and PAS was the product of Dai-ichi Seiyaku Co. The crystalline bovine serum albumin
was the product of Armour Laboratories. TB-004 was synthesized by the method described in the other paper (7). The absorption spectra were obtained with a Hitachi spectrophotometer.

RESULTS AND DISCUSSION

Metachromasy and its reversal

The absorption spectra of BCG in the free form and when bound to bovine serum albumin at pH 7.0 are shown in Fig. 1-a, 1-b and 1-d. The absorption maximum of BCG in the free state is at 610 m\(\mu\) and is shifted to 620-625 m\(\mu\) in the bound state. The molecular extinction coefficient, \(\varepsilon\), at 610 m\(\mu\) is \(3.00 \times 10^4\) in the presence of 0.015% albumin and this is about 65% of that without albumin. These data are in agreement with those by Rodkey et al. The decrease in absorbancy on binding is a function of the concentration of albumin, as shown in Fig. 2.

When a high concentration of PAS was added to the above metachromatic system, the spectrum became similar with that of BCG in the free state, as shown in Fig. 1-c. This reversal of metachromacy is a function of the concentration of PAS, as shown in Fig. 3. The reversing effect was expressed in terms of \(\Delta O.D._\text{a}\), an increase in absorbance at 610 m\(\mu\) of the BCG-albumin system containing PAS. It is shown that there was a half reversal when the concentration of PAS was \(5 \times 10^{-3}\text{M}\).

A comparison of the competitive ability of PAS with those of BA, SA and PABA against BCG is given in Table 1. The comparison was made at equal concentration of the competitors, namely \(5 \times 10^{-4}\text{M}\) and at pH 7.0. From the data given in the table, it

![Fig. 1. Metachromasy of BCG with albumin and its reversal by PAS. The concentration of BCG was \(5 \times 10^{-4}\text{M}\) in \(10^{-2}\text{M}\) phosphate buffer.](image-url)  
(a) : No albumin, (b) : 0.015% albumin added, (c) : 0.015% albumin plus \(2.5 \times 10^{-2}\text{M}\) PAS added, (d) : 0.05% albumin added.
is clear that the reversing effect of PAS was somewhat weaker than that of SA and stronger than that of PABA. This indicates an auxiliary role of the o-hydroxyl group in protein binding of PAS and SA, besides an essential role of the carboxyl group. It is also apparent that the introduction of an amino group into a BA derivative decreased its binding activity with albumin.

**TABLE 1.** Competition between BA derivatives and BCG at pH 7.0.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (M)</th>
<th>Δ O.D. at 610 mμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>0.005</td>
<td>0.026</td>
</tr>
<tr>
<td>PABA</td>
<td>↗</td>
<td>0.017</td>
</tr>
<tr>
<td>SA</td>
<td>↗</td>
<td>0.045</td>
</tr>
<tr>
<td>PAS</td>
<td>↗</td>
<td>0.033</td>
</tr>
<tr>
<td>TB-004</td>
<td>↗</td>
<td>0.067</td>
</tr>
</tbody>
</table>
**Dependence on pH**

In the absorption spectra of BCG, the difference in the heights of the absorption peaks in the absence and presence of serum albumin varied with pH as shown in Fig. 4. No measurements were made at below pH 6.0, since the interpretation of spectral changes in this dye was complicated by the ionization of the phenolic hydroxyl group. As is clear from the figure, the difference in absorbancy becomes smaller stepwisely with increase in pH from 6.5 to 12.0. In the former pH range the dissociation of the histidine residue occurred and in the latter range that of the lysine and arginine residues occurred. The difference in absorbancy disappears completely at above pH 12.0. These facts show that binding of BCG with albumin became weaker with increase in pH and that no binding occurred at above pH 12.0.

![Graph](image)

**Fig. 4.** Effect of pH on the BCG-albumin metachromasy. The concentrations of BCG and albumin were the same as in Fig. 1-b, but pH values were adjusted with NaOH. (a) : BCG alone, (b) : BCG and albumin.

A similar picture of the change in absorbancy with pH, except pH's below 9.0, has been obtained for the MO-albumin system by Klotz and the following conclusion is possible that the sulfonic group of BCG and the carboxyl group of PAS, BA, SA and PABA combine with positively charged nitrogen atoms of such basic amino acid residues as histidine, lysine and arginine of serum albumin under physiological conditions. However, it should be noted that the o-hydroxyl group in PAS and SA plays an auxiliary role in albumin binding through hydrogen bond formation.

**Binding of an SA derivative of a complicated structure**

As well as BA derivatives mentioned above, those of more complicated structures could be expected to bind with albumin. As an example, an SA derivative of the following structure, TB-004, was examined.
TB-004 was synthesized by Yamabe et al. (7) and was found to have a weak antituberculous activity by Ishida et al. (8). The data in Table 1 show that it had a high binding activity with albumin. This is considered due to its strong acidity as well as an azodye-like structure.

**Design of a PAS derivative with less protein binding activity**

On the basis of the above information about the binding sites of PAS on the albumin molecule, it appears that if the carboxyl group of PAS is converted to a non-ionizable group and another amino group is introduced to increase the basicity of the molecule, the affinity for serum albumin will be greatly reduced. However, the ability to chelate metal ions will be conserved and hence the antituberculous activity may also be conserved (9). Accordingly a new PAS derivative, aminobenzyl PAS was designed. The physico-chemical properties and antituberculous activity of this compound will be reported in the next paper.

**SUMMARY**

Metachromasy of the BCG-bovine serum albumin system was studied over a wide pH range. The results obtained indicate that the binding sites of BCG on albumin under physiological conditions are basic amino acid residues as lysine and arginine. Addition of benzoic acid derivatives, such as PAS, reversed the above metachromasy. It is thus concluded that the binding sites of PAS are the same as those of BCG. On the basis of this information, a new PAS derivative, aminobenzyl PAS was designed, and this is expected to show less protein binding activity.

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