Whether the effect is the result of an actual inhibition of aerobic metabolism or a confinement of the blood flow to non-nutritional vascular beds is not known. It appears, however, from *in vitro* experiments on smooth muscle, to be reported separately(16), that chlorpromazine in the concentrations used here *in vivo* abolishes completely the contractile response to vasoactive agents after cyanide poisoning of the respiratory enzymes of the muscle, as contrasted with only a lowering of the responsiveness to the same agents in normal smooth muscle treated with the same dose of chlorpromazine.

It is not possible to assess the potential therapeutic value of chlorpromazine for long term survival of extensively burned rats on the basis of the presented data. Apparently dose level and time of administration are of crucial importance and have narrow limits. The possibility exists that much lower doses of the drug at 24 hours after burn might preserve the early beneficial effect and thereby avoid the later deleterious effects. Experiments to test this possibility are in progress.

Summary. 1) The burned rat is extremely sensitive to changes in environmental conditions. 2) Fluid therapy substantially increases survival at 24 and 48 hours, whereas 10 day survival is only moderately increased. 3) The immediately lethal consequence of burn shock is nullified by proper dose levels of chlorpromazine. 4) The same dose of chlorpromazine has a deleterious effect later in the post burn course and increases mortality when given for 48 hours or longer.

The technical assistance of Miss Margaret Shepherd and Mr. Harold Silverman is gratefully acknowledged.

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Dissimilarity of Changes Induced in Absorption Spectrum of 2-(4'-Hydroxyphenylazo)-Benzoic Acid by Different Serum Albumins. (28319)

JAMES H. BAXTER

Laboratory of Metabolism, National Heart Institute, Bethesda, Md.

The pale yellow dye 2-(4'-hydroxyphenylazo)-benzoic acid has been widely employed in quantification of human serum albumin, utilizing the method of Rutstein *et al.*(1). The dye binds selectively to the albumin of human serum, whereupon its spectral absorption increases in the region of 480 mμ, under certain conditions in proportion to the albumin concentration(1,2). Employing this dye method with human albumin standards, we

obtained incorrect serum albumin values in dogs and rabbits. The present studies were done to investigate the reason for this occurrence. Serum albumins from different animal species are found to induce widely different changes in the absorption spectrum of the dye.

Methods and materials. Dye. The 2-(4'-hydroxyphenylazo)-benzoic acid, obtained from Dajac Laboratories, melted at 201-205°C and migrated as a single spot in paper chromatography using 24% acetic acid in toluene, saturated with water. *Albumins.* Fraction V's prepared by Cohn method 6 from sera of man,* cow, dog, horse, hog, rat, and rabbit were obtained from commercial sources and/or prepared in this laboratory. Albumin samples from man, dog, rat, guinea pig, and rabbit were also prepared by electrophoresis of serum on polyvinyl chloride followed by elution of the albumin peak in its entirety or in sections. Crystalline human and bovine albumins were purchased from Pentex, Inc. Most of the preparations contained 95-99% albumin, determined by paper electrophoresis. In addition to these relatively pure albumins, one or usually several samples of fresh or lyophilized whole serum were studied in the case of each of the 13 species discussed. Bound fatty acids(3) in the preparations amounted to about one mole per mole of albumin or less. Extraction of bound fatty acids(4) from a sample of bovine albumin and removal of small molecules from many of the preparations by dialysis did not influence the results. *Solutions of the dye and of the albumins* were made separately in 0.05 M phosphate buffer, final pH being accurately adjusted to 6.2. Before their 1:1 dilution by mixing as described below, all albumin solutions were 6 x 10^{-5} M, and the dye solutions ranged in concentration from 3 x 10^{-5} to 120 x 10^{-5} M. An exception was the frog serum which was diluted 1:10 without determining albumin content. The solutions were cleared by centrifugation if necessary.

Determinations of dye spectral changes induced by the albumins. Aliquots of each albumin solution were mixed in succession with equal aliquots of each dye solution. Then the absorbancy of each such mixture after deducting the absorbancy of the albumin alone, was compared over the range of 300-520 mμ with the absorbancy of an equivalent solution of the free dye. Thereby the changes in absorption of the dye solution caused by a certain part of the dye being bound by the albumin are determined in each case. *The quantity of bound dye* in many cases was determined in parallel experiments employing the equilibrium dialysis technic as used by Karush(5). Equal volumes of the dye and albumin solutions, instead of being mixed, were placed on opposite sides of a cellophane membrane and rocked until the dye had reached equilibrium. Then the concentration of dye bound by the albumin on one side of the membrane was calculated from the initial and final concentrations of free dye on the other side determined by reading the absorbancy of the dye solution at 348 mμ, allowing for the small quantity of dye adsorbed by the cellophane. The results were expressed as the average number of moles of dye bound per mole of albumin (ν).‡ Although the present study is primarily concerned with the gross

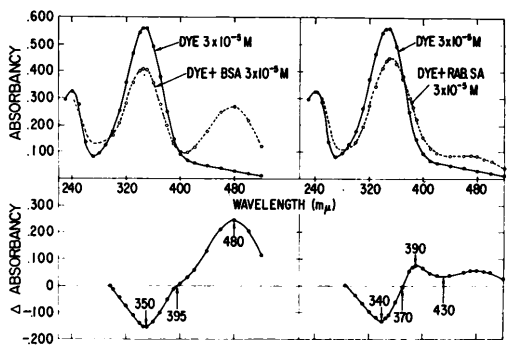

FIG. 1. Effects of bovine serum albumin (left) and rabbit serum albumin (right) on absorption spectrum of the 3 × 10^{-5} M dye solution.

absorption changes induced by the different albumins without adjustments for quantitative differences in dye binding by the different albumins, some of the binding data are presented.† *Spectral absorption determinations*

* Supplied through the courtesy of American National Red Cross. The preparations had not been heated or treated with stabilizers.

‡ The $\bar{\nu}$ used in this article will read ν.

† The albumin was confined to one-half the total volume of the system in the dialysis experiments but

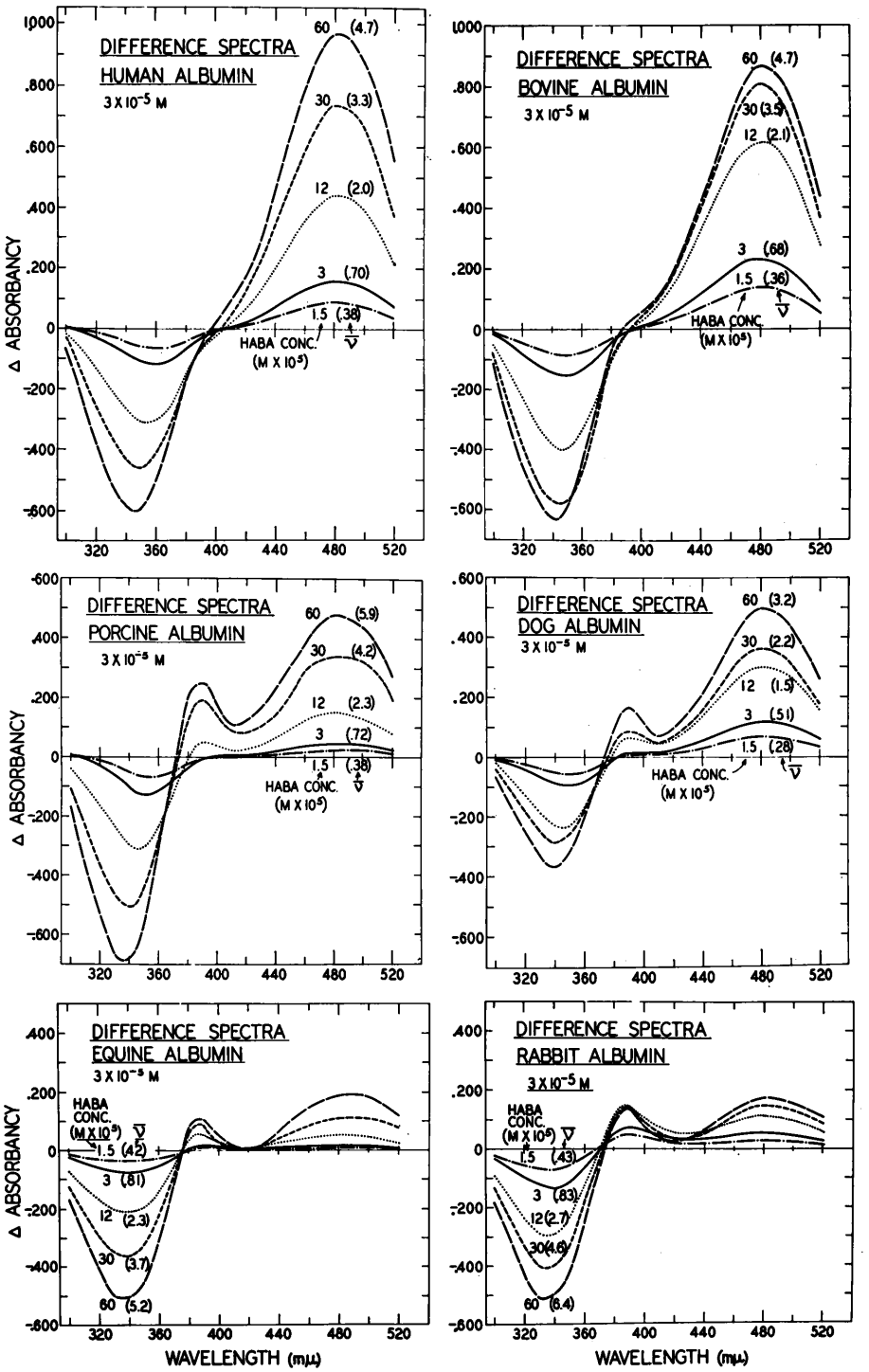

FIG. 2. Dye spectral absorption changes obtained by adding various Fr. V albumin preparations to dye (HABA) solutions of successively greater concentration. The relative quantities of bound dye are shown by the ν values.

were made with the model DU Beckman spectrophotometer, using matched quartz curvettes of 0.1-1.0 cm light path. Temperature of the solutions was maintained at 25°C throughout the experiments. It was established that absorbancy of the free dye adheres to Beer's law under the conditions of the study, the molar absorbancy index at 348 mμ being about 1.85×10^4.

Results. In the upper portion of Fig. 1, the absorption curve of 3×10^{-5} M dye solution alone is compared directly with that of the same solution containing successively 3×10^{-5} M bovine albumin and 3×10^{-5} M rabbit albumin. Absorbancy contributions of the albumins themselves have been subtracted, the dashed lines representing absorbancy of bound and residual free dye. It can be seen that bovine albumin causes a decrease in height and little change in wavelength of the 348 mμ dye absorption peak, together with the appearance of a large new absorption peak having a 480 mμ maximum. In contrast, rabbit albumin causes a shift in the 348 mμ peak toward the red end of the spectrum, with only a small increase absorbancy at 480 mμ. A much greater deepening in visible color of the dye solution occurs with bovine albumin by virtue of the greater 480 mμ absorption associated with it, therefore the effects of the two albumins can be easily distinguished with the unaided eye. The smaller increase in 480 mμ absorption induced by rabbit albumin is not attributable to a smaller quantity of the dye being bound by rabbit albumin, as will be seen below. Difference spectra are shown in the lower portion of Fig. 1. Compare particularly the respective negative maxima, isoabsorptive points (*i.e.*, the points at which the dye absorption is not changed by the albumins), and character of the 390 mμ regions.

In Fig. 2 are shown the changes in dye absorption obtained by adding different albumins always in 3×10^{-5} M final concentration to dye solutions of varying concentrations. Several conclusions are apparent from these data: (a) As the dye concentration increases, magnitude of the spectral deflections induced by each albumin increases. The relationships between $\Delta A_{480\ m\mu}$ and ν generally are approximately linear, the most marked exceptions perhaps being observed with bovine and equine albumins (Fig. 3). (b) As the magnitude of spectral deflections increases, the pattern of deflections changes somewhat in many cases. It appears from the curves in Fig. 2 that the greatest overall differences in the effects of the different albumins are seen with a dye concentration in the region of 3×10^{-5} M or less. (c) The spectral deflections obtained with the different albumins are all different, although the consistency with which the results can be obtained remains to be considered. Some of the curves obtained with the 3×10^{-5} M dye solution are compared directly in Fig. 4. It is evident that the differences in deflections at 480 mμ and elsewhere are by no means fully explained by quantitative differences in dye binding by the different albumins.

Many experiments have been made to determine whether the results described above can be consistently obtained. Each particular

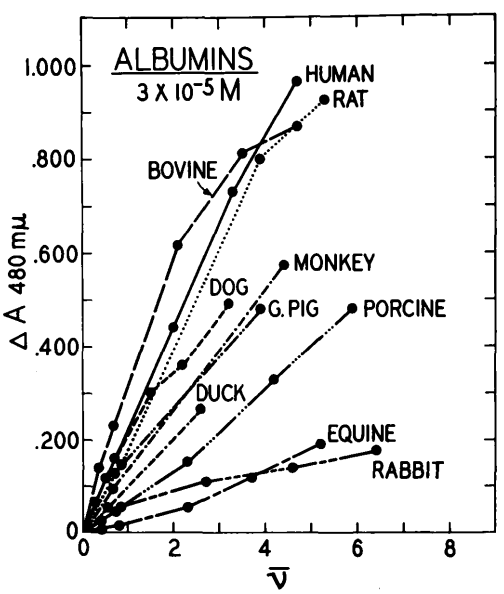

FIG. 3. Relationships between moles of dye bound per mole of albumin and magnitude of 480 mμ deflection for individual serum albumins.

not in the mixing experiments. The resulting 2-fold difference in albumin concentration in the 2 types of experiments did not, however, influence dye binding by the albumin. This was demonstrated by obtaining similar plots of ν *vs* free equilibrium dye concentration using 3×10^{-5} and 6×10^{-5} M albumin solutions in dialysis experiments.

TABLE I. Summary of Spectral Absorption Changes Induced in the 3 × 10^{-5} M Dye Solution by Different Serum Albumins.

Albumin (species) 3 × 10^{-5} M	Direction of 348 mμ peak shift†	Isosorptive point,‡ mμ	$\Delta A_{480\ m\mu}$
Monkey*	B	410	+++ (.070-.133; 4)§
Human	B	406	+++ (.115-.184; 14)
Porcine	B to 0	395; 380	++ (.042-.048; 4)
Bovine	(B)	396	++++ (.220-.242; 4)
Canine	0	385	+++ (.090-.124; 4)
Caprine*	(R)	385	+++ (.112; 1)
Ovine*	(R)	383	+++ (.112; 1)
G. pig	(R)	385	+++ (.130-.213; 4)
Equine	(R)	378	+ (.009-.022; 4)
Rat	R	374	+++ (.147-.216; 15)
Rabbit	R	370	++ (.048-.054; 5)
Duck	R	368	++ (.052-.055; 2)
Frog*	Very slight changes		(<.010; 2)

* Studies were limited to sera.
† B = blue shift; 0 = little or no shift; R = red shift. Parentheses indicate a slight shift.
‡ Variable by 2 to 5 mμ in either direction.
§ Range of values, followed by number of preparations studied.

preparation of albumin was found to yield highly reproducible results. Different preparations of the individual albumins produced changes which were likewise nearly always similar in pattern—duplicating the patterns that have been illustrated—but which were often somewhat variable in magnitude of the peaks. Based on results with all of the albumin preparations studied, the major absorption changes consistently produced in the 3 x 10^{-5} M dye solution by the different albumins are summarized in Table I. Magnitude of the 480 mμ deflections most often fell near the midpoints of the ranges shown, and neither serum nor any other particular type of preparation produced results which consistently deviated in one direction from the others.

Discussion. The observations indicate that the different serum albumins induce spectral absorption changes in the dye which vary both in magnitude and pattern, but that the individual albumins induce changes which are reasonably consistent and distinctive. Previous workers have made somewhat similar observations using other dyes under much more limited conditions(6,7). Possibly some indications of species relationships can be seen in the present results. The differences in effects of the different serum albumins presumably are related to differences in structure of the albumins.

It has been assumed in the foregoing presentation that all absorption changes in the dye-albumin mixtures are attributable to absorption changes in the dye. This may be an oversimplification, since changes in absorption of the albumin may also occur as a result of the dye-protein interaction and contribute slightly to the absorption changes observed in the 300-340 mμ region.

The variations in results obtained with different preparations of the individual albumins are not fully understood. Inaccuracies in estimating true albumin content and possible alterations induced in preparing and handling the albumin preparations may have accounted for a part of the variation. In addition, it seems likely that there is some natural variability in the "albumins" prepared by the different methods and even in those prepared by repetition of the same method. The purpose of employing the variety of preparations was to assure that the species differences in albumin-induced spectral changes are not artifactitious and to determine to what extent the results are influenced by the methods employed in preparing the albumins. If it can be determined which preparations most nearly represent pure and unaltered albumin (from the standpoint of the dye spectral changes induced by them), use of such preparations might be expected to afford less variable and more definitive results.

The increases in magnitude of spectral deflections obtained in this study by increasing dye concentration should not be confused with the increases in magnitude which may be obtained by increasing albumin concentration. One is caused by a greater quantity

of dye being bound per molecule of albumin, whereas the other is attributable to formation of greater a quantity of dye-albumin complex of decreased dye:albumin molar ratio. The changes in patterns of deflections observed here with changes in dye concentration evidently are related to the number of dye molecules bound per molecule of albumin. Whether the changes are dependent on differences in character of the successive albumin binding sites or on some other mechanism is not clear.

These results are of interest as a presumed example of changes occurring in the absorption spectrum of a small molecule dependent on the fine structure of the macromolecule to which the small molecule is bound. Slight changes occur in the absorption peaks of a number of dyes when the dyes become bound to albumin(8); however, studies in progress indicate that to develop a new absorption peak of the type noted here in the presence of albumin, a dye of highly specific structure is required. In addition to their possible theoretical significance, the results afford a means of quickly distinguishing between certain albumins. They also afford information which has important bearing on quantitative determination of serum albumins employing methods utilizing this dye. It is evident that such determinations should usually be based on albumin standards obtained from the same species as the albumin being determined. It is inferred that the method is relatively unsatisfactory for quantification of albumins which produce small 480 mμ absorption changes.

Summary. Addition of different serum albumins to solutions of the dye 2-(4′-hydroxyphenylazo)-benzoic acid caused widely different changes in the absorption spectrum of the dye. The changes differed in pattern as well as in magnitude, being largely independent of the quantitative differences in dye binding displayed by the different albumins. Different preparations of the individual albumins induced dye absorption changes sufficiently consistent and distinctive that each of the albumins could be distinguished from most of the others.

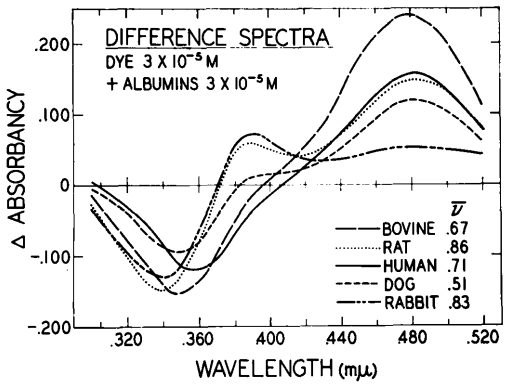

FIG. 4. Comparison of absorption changes induced in the 3 × 10^{-5} M dye solution by various serum albumins. Concentrations shown here and in other figures are those present after mixing dye and albumin solutions. Relative quantities of bound dye are indicated.

I am grateful for the assistance of Mr. Carlos Schultz.

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