FLUORESCEIN-CONJUGATED BOVINE ALBUMIN

Physical and Biological Properties*

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Plates 3 to 5

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The potential usefulness of optically detectable plasma proteins as a means of studying capillary permeability, especially by microscopic methods, led to the present efforts to prepare fluorescein plasma protein conjugates and to characterize some of their physical properties. Although Coons *et al.* (1, 2) demonstrated that the specific immunological reactivity of proteins is not destroyed by fluorescein conjugation according to their method of preparation, it is possible that alterations in molecular configuration, size, and shape exist, which while not affecting antigen-antibody union could seriously detract from its usefulness as a tool for studying transcapillary exchange of plasma proteins. This portion of the study was conducted primarily to ascertain to what extent, if any, the process of fluorescein conjugation altered the structure of the native starting materials.

The projected application of fluorescein-conjugated bovine albumin (BAC) as an indicator of permeability requires the fulfillment of additional criteria that apply to protein labeling in general, and that are identical to those proposed by Sterling (3) for I^{131}-labeled albumin; *viz.*, that the fluorescein-labeled albumin (1) behave *in vivo* like native albumin, and (2) remain labeled *in vivo* throughout the existence of albumin as an intact molecule. The experiments and observations described in this part of the report represent a study of the biological properties of BAC, designed in part to ascertain the extent to which the above criteria are fulfilled and also to indicate some specific areas of application to fundamental problems involving the transcapillary passage of plasma proteins.

Preparation of Fluorescein Protein Conjugates

The fluorescein crystalline bovine albumin conjugates (BAC) were prepared in accordance with methods described by Coons *et al.* (1, 2) modified to use a 2 per cent

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solution of crystalline bovine albumin (BA), and employing in the several preparations varying amounts of aminofluorescein per gram of protein (50, 100, 200, and 300 mg.).

The resulting conjugate mixture was filtered through filter-cel, dialyzed 6 days against saline and 2 additional days against distilled water, then lyophilized. All operations were carried out at 2°C. The final product was a fluffy, orange-yellow, water-soluble, yellow-green fluorescent substance. Based on protein used, yields of approximately 80 per cent were obtained regardless of the ratio of protein to aminofluorescein.

The same method of preparation was employed for conjugating fluorescein to rat, rabbit, and guinea pig whole plasma proteins and guinea pig serum albumin. These conjugates were not characterized physically; however, they could not be differentiated from the bovine albumin conjugates by inspection, water solubility, nor fluorescent hue and intensity.

Physical Characterization Studies

Electrophoretic studies were performed with BAC 5 at pH = 4.15 and pH = 4.55 in 0.10 ionic strength acetate buffers in a conventional Tiselius assembly (4) equipped with a cylinder lens optical system (5, 6). In a sedimentation experiment BAC 5 was centrifuged in 0.15 M NaCl at 50,400 R.P.M. in a Svedberg oil turbine ultracentrifuge. Intrinsic viscosity determinations (7) (0.15 M NaCl, pH 5.2, 37.0 ± 0.05°C., Ostwald viscometer) were made on BAC preparations 5, 6, 7, 8. Within the limits of error of the method, the intrinsic viscosity (H) of BAC 6, 7, 8 was identical with that of bovine serum albumin ($H = 0.042$ dl./gm.). BAC 5 had a slightly lower value, $H = 0.040$ dl./gm., suggesting that some molecules of BAC 5 are either more symmetrical, or less hydrated, than the BA molecules.

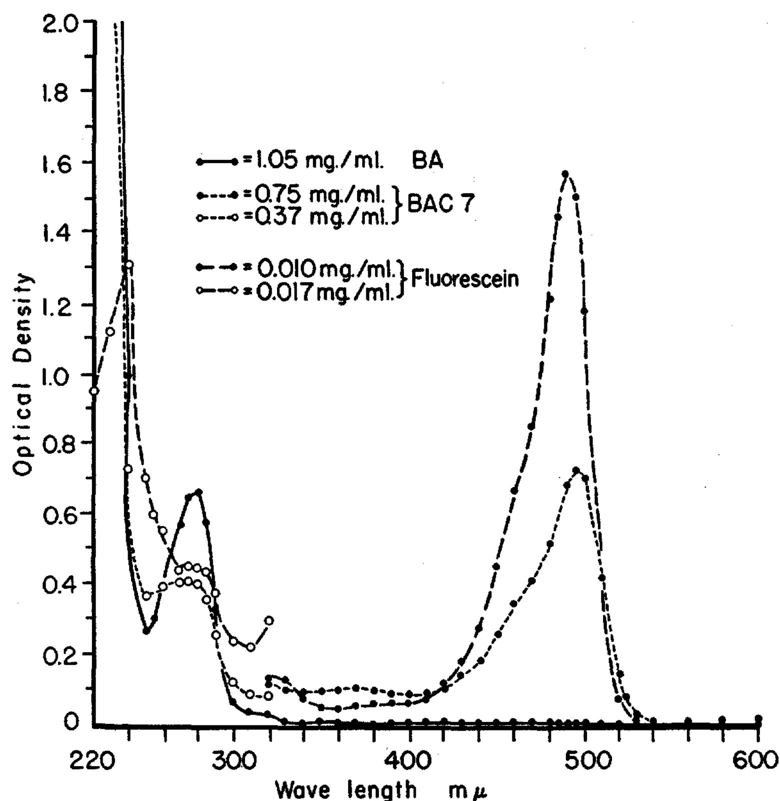
The sedimentation patterns of BAC 5 indicate that the sedimentation behavior is essentially unaltered by fluorescein conjugation, although a small shoulder on the leading edge of the peak suggests that a fraction (< 10 per cent) of the molecules in the preparation have a lower frictional coefficient (f/f_o) than does the BA molecule (8), in support of the intrinsic viscosity measurements.

The isoelectric point ($pI_e = 4.58$) of BAC 5 differs by 0.13 pH unit from that of BA ($pI_e = 4.71$) suggesting an average of 1.6 molecules of fluorescein per molecule of albumin. Electrophoretic spreading experiments indicate that the conjugated protein is only slightly more heterogeneous than the original albumin, supporting the evidence that one or two molecules of fluorescein are attached to each protein molecule.

On the basis of the intrinsic viscosity measurements, BAC 6, 7, 8 were used in biological experiments since these preparations appeared to be the least altered with respect to molecular size and shape.

Spectral Absorption and Fluorescence Emission

Spectrophotometry.—Comparison was made of the spectral absorption curves of sodium fluorescein, BA, and BAC, in a solvent mixture of 90 per cent distilled water and 10 per cent phosphate buffer adjusted to a pH of 7.2. Although minor differences are noted, no new absorption peaks are evident in the BAC absorp-



TEXT-FIG. 1. Representative spectral absorption curves of solutions of sodium fluorescein, BA, and BAC.

tion spectrum that are not present either in the sodium fluorescein or BA absorption curves. In the ultraviolet region, the BAC absorption curves show features characteristic of both fluorescein and BA curves and are considered to represent resultants of simultaneous absorption by the fluorescein moiety and the specific absorbing amino acids of BA (Text-fig. 1).

The most striking differences in the absorption properties of the three types of substances studied are to be found in the visible region of the spectrum at

λ 490 to 495 mμ, the absorption peak of fluorescein. Of the protein compounds studied, only BAC absorbs appreciably in this band (Text-fig. 1). On the basis of the absence of absorption by BA, it was assumed that the structure responsible for the specific absorption of BAC at λ 490 to 495 mμ is the fluorescein moiety. Since the concentrations of the sodium fluorescein solutions bear a linear relationship to their optical densities, it appeared likely that the concentration of BAC-fluorescein could be computed from the known extinction coefficient of fluorescein at λ 490 mμ. This was done for three samples of BAC prepared with

TABLE I

Properties of BAC Obtained by Optical Analysis

BAC preparation	Extinction coefficient	Molar ratio Mols BAC-fluorescein*/mols BAC‡		Aminofluorescein
		Fluorophotometric	Spectrophotometric	
	(liters/mol) × *10*[3]			*mg./gm. BA*
7	67.3	1.41	1.37	50
5	—	—	Approximately 1.6§	100
6	89.3	0.79	1.71	200
8	99.7	1.54	1.92	300

* Experimentally derived, see text.
‡ Computed; molecular weight BAC = 70,000.
§ Determined by electrophoretic analysis.

varying amounts of aminofluorescein (Table I), utilizing a modification of the Bunsen-Roscoe equation, *viz:*

$$E \text{ (sodium fluorescein)} = \frac{D}{C^1 d}$$

$$C \text{ (BAC fluorescein)} = \frac{D}{E}$$

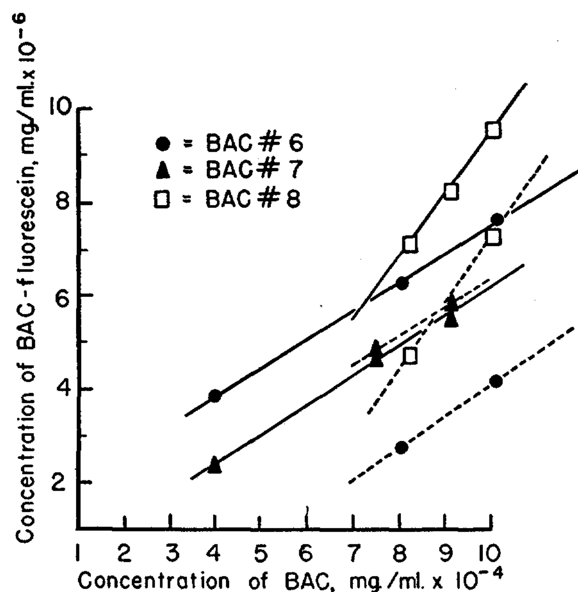
in which E is the extinction coefficient of sodium fluorescein, C is the concentration of solute in millimols per liter, D the optical density at λ = 490 mμ, and d = 1.0, the thickness of the absorption cell in centimeters. When the concentration of the BAC fluorescein is plotted against the concentration of BAC, a linear relationship is obtained, the curves of each BAC preparation differing from each other either in slope or intercept (Text-fig. 2).

The representative values for the extinction coefficients of three BAC preparations (Table I) can only be regarded as approximations since relatively few analyses were made. Yet the progressive increase in extinction coefficients

[1] Concentration calculated in terms of fluorescein base.

rougly parallels the quantities of aminofluorescein used in the several BAC preparations, which suggests the possibility that larger amounts of label can be coupled to BA by utilizing larger quantities of aminofluorescein since yields were not materially changed.

Fluorophotometry.—Aliquots of the solutions used in the spectrophotometric analyses were placed in 1 cm. square corex cuvettes and inserted in the cuvette holder of a fluorophotometer. Fluorescence emission, converted to photocurrent,



TEXT-FIG. 2. BAC-fluorescein concentrations, expressed in terms of equivalent free fluorescein concentration, determined spectrophotometrically (solid lines) and fluorophotometrically (dash lines).

was read from a galvanometer. These values were converted to equivalent fluorescein concentration from previously determined curves relating known fluorescein concentrations to galvanometer units. Control blanks, whose values were subtracted from the BAC readings, consisted of BA solutions of approximately the same concentrations as those of the BAC solutions.

BAC-fluorescein concentrations for varying concentrations of three different BAC preparations determined fluorophotometrically are listed in Table II, and are graphically represented in Text-figure 2. It is apparent that while the two optical methods provide parallel relative BAC-fluorescein concentration values, they may not yield similar absolute values. Thus, the fluorophotometrically determined BAC-fluorescein concentrations of preparations 6 to 8 are considerably lower than when determined spectrophotometrically, yet both meth-

ods yield approximately the same value for preparation 7 (Fig. 2). The lack of agreement between the two methods may be due to differences in absorptive and emissive properties of the conjugates.

For purposes of computing the molar concentration of BAC-fluorescein undoubtedly the absorption criterion is preferred. However, in many of its projected applications, the prime value of BAC derives from the intensity of its fluorescence. In this connection, fluorophotometric analyses provide a correct index of *effective* BAC-fluorescein concentration, which permits the BAC preparations, on an equimolar basis, to be ranked (decreasing order) 8, 7, 6, whereas

TABLE II

Estimates of BAC-Fluorescein Concentration

Preparation	BAC-fluorescein concentrations		BAC concentrations
	Fluorophotometric	Spectrophotometric	
	(gm./ml.) $\times 10^{-6}$	*(gm./ml.)* $\times 10^{-6}$	*(gm./ml.)* $\times 10^{-4}$
BAC 6			
1	2.75	6.24	8.05
2	4.15	7.61	11.15
3	—	3.83	4.00
BAC 7			
1	4.8	4.65	7.47
2	5.8	5.51	9.15
3	—	2.43	4.00
BAC 8			
1	4.75	7.11	8.20
2	7.3	9.60	10.00
3	—	8.22	9.10

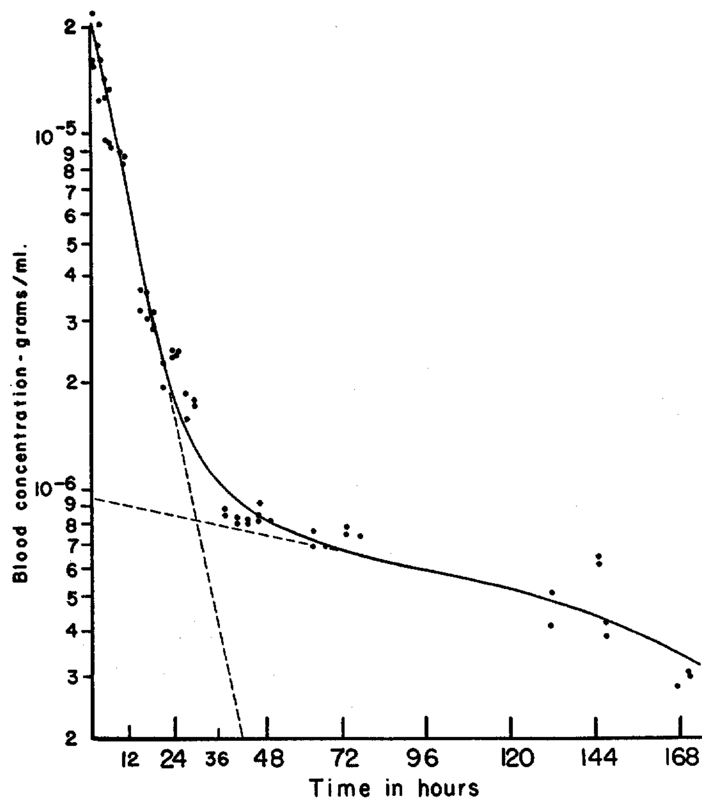
they rank 8, 6, 7, spectrophotometrically. It is interesting to note that the binding of fluorescein in BAC 5 yields a molar ratio of approximately 1.6 (Table I) as estimated from electrophoretic studies, agreeing closely with other members in the series ranked according to aminofluorescein content, but analyzed optically.

These results suggest that fluorescein-conjugated bovine albumin and the native starting material are sufficiently interchangeable substances in terms of their pertinent physical properties to warrant the use of these conjugates for experiments concerned with estimating the permeability of capillaries to circulating proteins.

Disappearance Rates of BAC

From the Circulation.—Recent investigation into the *in vivo* properties of plasma proteins and plasma protein fractions lightly labeled with isotopic

iodine (3) or carbon (9) has provided a basis of reference for comparing proteins labeled with other moieties. The disappearance rate of BAC from the circulation of intact rats was determined by computing the deficit of blood conjugate at various times after a single intravenous injection of BAC (25 mg./100 gm.). Postinjection blood samples were obtained at least 3, and in most instances 5 to

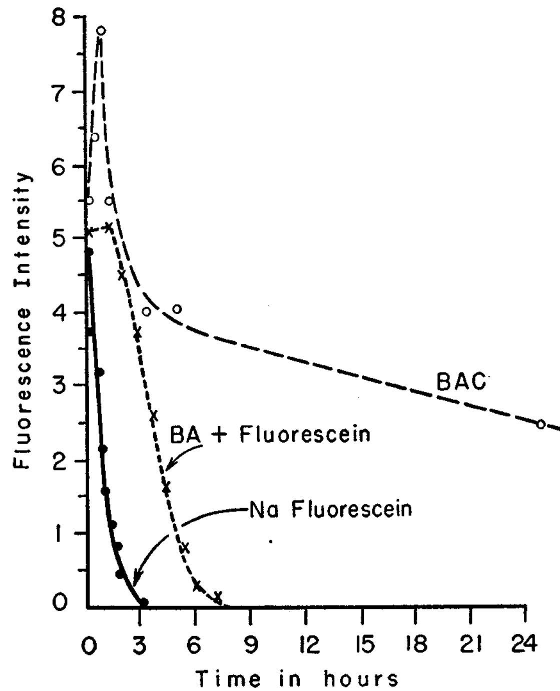


TEXT-FIG. 3. Disappearance rate of BAC from the circulation of rats. Concentration of BAC in the blood plotted (semilogarithmically) as a function of time.

6 times from each rat over a period of 12 days. A time-concentration curve on semilog plot (Text-fig. 3) shows an initial steep almost linear component through the 20th hour having a half-life of 6.4 hours. From the 20th to the 65th hour there is a gradual non-linear decline in the rate of conjugate disappearance, followed by a linear decrease to about the 160th hour with a calculated half-life of 5.3 days. These values are in reasonable agreement with those of Abdou and Tarver (9) who obtained half-lives of 8 hours and 3.1 days for the rapid and slow components, respectively, of the serum protein label disappearance curve in rats. The somewhat longer disappearance rate for the initial period obtained

by these investigators may be due in part to the fact that the globulins as well as the albumins of the plasma contained the C^{14} label.

Additional comparisons of circulatory protein disappearance rates from the literature are made difficult because of species difference, the rat having a faster turnover rate than that of other species studied (3). In general, BAC disappears from the circulation of rats in a manner similar to that of fluorescein-conjugated



TEXT-FIG. 4. Absorption curves of BAC, sodium fluorescein, and fluorescein albumin mixture from the abdominal skin of rabbit.

homologous plasma proteins and intrinsically labeled C^{14} serum proteins, indicating that the fluorescein-protein coupling is stable *in vivo*.

Absorption from Skin.—Despite the fact that BAC showed little tendency to dissociate during prolonged dialysis, the strength of the fluorescein-protein bond subjected to tissue fluid environment remained to be tested. Previous measurements of clearance rates of intradermally injected sodium fluorescein solutions in rabbits showed that complete absorption of the dye occurred within 3 to 4 hours. When sodium fluorescein dissolved in bovine albumin solutions of 0.25, 1.0, and 3.0 per cent concentrations was injected intradermally in 0.1 cc. volume, complete fluorescein absorption was delayed by approximately 3 to 5 hours. Following intracutaneous injections of BAC in comparable concentra-

tions, the conjugate was absorbed very slowly, in days rather than hours (Text-fig. 4). In fact, traces of label were still detectable in the skin 16 days after injection. Presence of the conjugate in the skin at this time was confirmed histologically from biopsy specimens examined with the fluorescence microscope.

Had fluorescein dissociated from the conjugate the absorption time should have approximated that of the fluorescein-protein mixtures. In view of the fact that this was not the case, it is evident that BAC under these conditions does not dissociate to give free fluorescein or fluorescein-labeled split products of crystalloid dimensions.

Localization of BAC in the Tissues

The distribution of extravascular BAC was studied by examining representative tissues histologically in the fluorescence microscope. The tissues and organs taken from rats sacrificed at intervals between 2 hours and 23 days after a single intravenous injection of BAC, were prepared for sectioning by, (*a*) CO_2 freezing with and without fixation, (*b*) CO_2 freezing according to the Adamstone-Taylor technique (10), (*c*) freezing-dehydration (11, 12), and (*d*) conventional fixation and imbedding in paraffin. Parallel treatment of tissues from rats intravenously injected with equivalent amounts of BA provided control histological preparations. It soon became apparent that the cytology, the fluorescence intensity and hue of the label, and its diffusibility could be altered by relatively minor modifications of histological preparation. It is important to emphasize that histological analysis by fluorescence microscopy, virtually a new procedure, when directed at complex situations whose components are either unknown, unevaluated, or both, and without benefit of a time-tested background of experience, requires considerable caution in interpretation.

Since a systematic study of the factors responsible for possible artifact was not attempted at this time, the localizations of label described below were based upon the positive criteria that labeled protein gave a characteristic saturated yellow-green fluorescence of fluorescein of relatively high intensity which did not fade on exposure to UV, and which did not appear in control sections. Fluorescent hues of orange, yellow, or unsaturated yellow-green were frequently seen in control sections, and in some cases were strongly developed or repressed by the method of preparing the tissues. However, in no case did the tissues of untreated or BA-treated rats exhibit autofluorescence of a *saturated* yellow-green color, characteristic of BAC or fluorescein.

Intracellular.—Tissue phagocytes were very active in concentrating the label in their cytoplasm in the form of tiny globules or granules. The Kupffer cells of the liver provided the most striking example of this property (Figs. 1 to 6). The label was found in the cytoplasm of these cells (Fig. 6) within 2 hours after injection of the BAC and reached what appeared to be maximum concentration

within approximately 6 hours, persisting for several days after other intracellular sites had lost the label. Cells with the morphological appearance of fibroblasts in skeletal and cardiac muscle (Fig. 14) and multilobed granulocytes in the connective tissue stroma of blood and lymphatic vessels also showed a preferential ability to concentrate the conjugate within their cytoplasm. Label was consistently absent from the parenchyma and interstitial fluid of the brain, despite its heavy concentration within the cerebral blood vessels. A striking finding was the heterogeneous distribution of label both among tubules and within the individual cells of cross-sections of single tubules. Label-bearing tubules were found in groups or clumps, separated by groups of tubules which did not contain the label, giving the impression that only certain nephron units were functional and others were not (Fig. 7). Further, certain cells within a given tubule were seen to have strongly green fluorescent cytoplasm, whereas an adjacent cell would have only a pale yellow cytoplasm or even exhibit the blue-white autofluorescence characteristic of control tissue (fig. 9). Smetana (13) observed similar renal localizations of dye azo serum proteins in rats and mice.

Extracellular Localizations.—The label of the conjugate was evident within the lumina of blood vessels, particularly during the first 12 hours postinjection, declining in intensity until it was no longer perceptible after approximately 36 hours. At this time the blood concentration of conjugate had fallen to approximately 1 γ/cc., which represents the threshold concentration for its perception under the conditions of observation. On this basis, BAC is a more sensitive tracer than dye azoproteins (13, 16).

Although the label was rarely seen in the interstitial fluid of glandular organs, especially the liver and spleen, it was readily demonstrated in the interstitial spaces of skeletal muscle, appearing to outline the connective tissue investment of the muscle fasicules (Fig. 15). In cardiac muscle, the interstitial localization of label was particularly evident at the endocardial surface, in the papillary muscles, and subepicardially (Fig. 13).

The most interesting extracellular location of label was its occasional presence in the lumina of the proximal kidney tubules (Figs. 10 and 11) and its consistent absence from the lumina of the collecting tubules (Fig. 12). Since the label can be detected in the urine, its absence in the collecting tubules suggests that its concentration there is below perceptible threshold. As indicated by others, this casual observation of a difference of concentration of label at the two ends of the nephron may be interpreted as evidence for tubular absorption of protein, especially in view of the intracellular localization of label in some of the proximal tubules, but not in the collecting tubules.

One of the most constant findings was localization of the label in relatively high concentration on vascular endothelium, most striking up to 18 hours postinjection. In heart, glomerulus, spleen, and skeletal muscle, the affinity of label

for endothelium made the vascular arborization traceable by its delicate green outline (Figs. 8 and 16). Resolution was never sufficient to accurately determine whether the label had entered endothelial cells or was merely adherent to their surfaces. However, evidence from other sources strongly indicates that adsorption is probably the responsible mechanism.

Cytoplasmic vs. Nuclear Localization.—A characteristic negative finding was the absence of label in the nuclei of those cells in which it was definitely localized in the cytoplasm; *e.g.*, Kupffer cells (Figs. 2 and 6) in agreement with others who have used fluorescein as well as azo-coupled proteins (12, 13, 16, 17). In the light of the recent observations of Coons *et al.* (14) that fluorescein antibody label is frequently found within the nuclei of fibroblasts and hepatic and renal tubule cells of mice, we carefully reexamined these sites in our own preparations but were unable to make a similar observation. Their use of bovine albumin as an antigen, and the description of label localization in the nuclei of cells whose cytoplasm was free of label only serve to emphasize the disparity of results. Coons (15) believes that the most likely explanation may be one of sensitivity arising from differences in the preparation of sections, in equipment for microscopy, or in the employment of the fluorescein label. In view of the very strong intensity of label fluorescence in the cytoplasm of the Kupffer cells without evidence of its presence in the nuclei (Fig. 6) this explanation is not wholly compelling. Although an acceptable explanation for these differences is not immediately apparent, it seems reasonable to implicate the methods of introducing labeled protein since they differ considerably, being incorporated *in vivo* in the case of BAC and involving *in vitro* coupling of intracellular antigen with labeled antibody in Coons's experiments. It is possible that this difference throws some light on the permeability of the nuclear membrane since Coons *et al.* may have been detecting immunologically active fragments of the injected protein molecules (14, 15). Equally plausible, however, is the possibility that antigen protein or immunologically active fragments redistribute themselves within the cell during the thawing and fixing steps prior to application of the labeled antibody. In other respects, localizations of BAC with regard to cell types, intensity, and persistence of fluorescence conform reasonably well with those reported by Coons *et al.* and others (13, 14, 16).

Although the great bulk of the microscopic observations described were made from the tissues of rats injected with BAC, the results were essentially qualitatively the same in four rats injected with homologous fluorescein plasma protein conjugate, confirming the observation that native and foreign proteins are treated similarly *in vivo* (16).

It is believed that fluorescein conjugated to plasma proteins or its separate fractions provides a reliable optical label for studying *in vivo* histological localization, and by inference the fate and metabolism of plasma proteins. In this

connection it is interesting to note that the cell types which have been implicated in protein metabolism; *viz*, reticuloendothelial cells, liver, and kidney, are the very sites which strikingly localize the label.

Capillary Permeability

Cutaneous Thermal Burn.—In order to assess the value of the conjugate as an indicator of increased vascular permeability it was administered to animals which were later subjected to localized cutaneous thermal burns. BAC was injected intravenously (25 mg./100 gm.) into rats whose abdomens had been previously depilated. Approximately 20 hours later the abdominal skin was without visible evidence of green fluorescence. A constant temperature (65°C.) water thermode was placed in contact with two different areas of abdominal skin of each rat for variable intervals ranging from 10 to 60 seconds. Within 30 to 60 minutes the burned sites became discretely and uniformly deep green fluorescent under UV illumination. The intensity of the fluorescence appeared to parallel roughly the severity of the burn in those sites which later proved to be second degree burns of variable extent.

From these experiments the following conclusions were drawn: (1) the normal cutaneous vessels of the rats' abdomens are relatively impermeable to circulating BAC, (2) BAC remains in the vascular compartment in detectably high concentration for at least 20 hours, and (3) gross evidence of protein leakage indicative of locally increased capillary permeability is readily detected following local burn.

Observations on the Mesenteric Circulation.—The mesenteries of frogs and rats were prepared for microscopic observation in a manner similar to that described by Chambers and Zweifach, but adapted for the fluorescence microscope. After assessing the status of the mesenteric circulation under white light illumination, BAC or rat fluorescein plasma protein conjugate was injected, intracardially in the frog, or intravenously in the tail of the rat (25 mg./100 gm. of a 10 to 20 per cent solution).

Under UV illumination, prior to the injection of conjugate, the blue-white autofluorescence of the larger vessels faintly outlined the vascular arborization against an almost black background. Shortly after the injection of the conjugate the large vessels became distinctly visible resembling green tubes with dark central cores, readily identified as the plasma sleeve and the opaque axial stream of red cells, respectively (Figs. 17 and 18). Vessels of capillary dimensions soon became perceptible as fine green threads of relatively low fluorescent intensity in which occasional shadows of red cells could be seen traversing their length (Fig. 17). The minute vessels retained their sharp green fluorescent outline for approximately 15 to 30 minutes, after which time their edges became progressively less distinct as the conjugate diffused into the tissue spaces. 2 to 3 hours

from the time of conjugate injection the interstitial space was diffusely illuminated with the yellow-green fluorescence of the conjugate, yet the intravascular conjugate concentration was still perceptibly higher than that of the tissues (Figs. 17 and 18), indicative of the continued existence of a blood to tissue concentration gradient.

When vessels of capillary dimensions were gently stroked, jabbed or pricked with microneedles, the outward passage of conjugate was dramatically increased, indicating that injury of their walls and adjacent tissues is associated with readily demonstrable evidence of increased permeability. Unfortunately technical obstacles to photomicrography of such dynamic events under conditions of low illumination have not yet been solved.

The above sequence of events contrasts sharply with that seen following the intravascular injection of a solution of the crystalloid sodium fluorescein, which on reaching the mesenteric vessels swiftly outlines the arteries, capillaries, and veins in an interval of 1 to 2 seconds, followed by prompt diffusion into the tissue spaces. Within 2 to 5 minutes the vessels appear as dark shadows in a brightly yellow-green fluorescent field, indicative of a reversal of concentration gradient from tissue fluid to blood. The rapid diffusion of dye precludes the possibility of studying the effects of locally induced mild tissue injury by available techniques.

It is evident from these observations that the conjugate diffuses through the capillaries in a manner anticipated for particles of colloidal dimensions. Its slow diffusion rate through the capillary walls and its progressive accumulation in the tissue spaces suggest the possibility of quantitative photometric measurements of label concentration in a given tissue area as a function of time. The accumulation rate of label in a fixed tissue site may prove to bear a relationship to the permeability of capillaries for the labeled protein. However, it is recognized that conditions in the extravascular space such as lymphatic drainage, and differential solubility of the labeled protein in the aqueous-colloid phases of ground substance, may introduce additional factors in the correlation of photometric data with permeability of the capillary wall *per se*. In their broadest interpretation, these observations imply that the visualization of plasma proteins by virtue of their fluorescent label makes available for the first time the application of microscopic methods for studying directly the transcapillary passage of circulating plasma proteins, with potentialities for quantitation.

Antigenicity Studies

It has been demonstrated that the conjugation of fluorescein with antibody does not alter its immunologic specificity (2). However, the evidence was based upon an *in vitro* coupling reaction. In order to determine whether the process of conjugation altered native plasma protein sufficiently to make it antigenic,

whole guinea pig serum and guinea pig serum albumin were conjugated to fluorescein in the manner previously described. Approximately 3 weeks following sensitizing doses of the above conjugates, shocking doses were administered intracardially. No evidence of anaphylaxis was observed among twelve animals injected with guinea pig albumin conjugate. However, the guinea pig whole serum did possess moderate antigenicity. One of twelve sensitized animals died in anaphylactic shock, four showed mild anaphylactic symptoms but recovered, and the remaining seven guinea pigs showed no signs of anaphylaxis. Azo dye-protein coupling, by contrast, renders native protein, including albumin, antigenic.

DISCUSSION

A labeled plasma protein intended for use in investigations of capillary permeability and tissue distribution should conform as closely as possible to the native plasma protein from which it is derived in order to simplify interpretation of experimental findings. Aside from possible alterations of reactive groups, change in the size, shape, and charge distribution of a labeled protein are the most significant factors which might distort or invalidate its identity with its native homolog in terms of the pore theory of transcapillary passage. Intrinsic viscosity and sedimentation velocity studies clearly show that significant changes in size and shape have not occurred as a consequence of fluorescein conjugation. Electrophoretic studies indicate that the process of conjugation has not altered greatly the charge distribution. As a first approximation it would appear that fluorescein molecules are rather homogeneously distributed among the protein molecules. The results of spectral absorption studies are in agreement with those of electrophoresis, and in addition show that fluoresein is the absorbing moiety in the visible region of the spectrum, the extent of absorption being proportional to the amount present.

As evidence that fluorescein-labeled plasma protein behaved *in vivo* in a manner similar to its native precursor, it was shown that (*a*) its disappearance rate from the circulation corresponded with that determined by the use of isotopically labeled plasma proteins, and (*b*) its localization in the various tissue sites is consonant with the findings of others using the same and different types of labels, with a few exceptions and additions. It is important to emphasize in this connection that fluorescein protein conjugates differ from dye azoproteins in that the former show less evidence of denaturation than the latter on the basis of physical and antigenicity studies. The slow disappearance rate from the skin, and slow diffusion through the living mesenteric capillaries further demonstrate the colloidal behavior of the conjugate.

The second criterion alluded to in the opening paragraphs requires that the protein retain its label *in vivo* throughout the existence of the protein as an in-

tact molecule. This problem has been treated in detail by Kruse and McMaster (16). Their analysis and conclusions regarding the blue dye (echt-säure-blau) azo coupled to γ-globulin seem particularly applicable to fluorescein protein conjugates. Fluorescein is known to be predominantly bound to lysine (18) which, according to Schoenheimer, shows the least propensity among the amino acids for participating in the metabolic pool. Therefore, it is reasonable to believe that if label shifting does occur, it should be minimal under these circumstances.

Sodium fluorescein has been employed in experiments similar to those described in this report with results differing greatly from those yielded by fluorescein protein conjugates. The differences are essentially those predictable for the *in vivo* behavior of crystalloidal *versus* colloidal substances as exemplified by their disappearance rates from the skin (Text-fig. 4) and mesenteric capillaries. Free fluorescein would undoubtedly reveal itself through its crystalloid behavior. Since such patterns were never observed with fluorescein protein conjugates, it is believed that free fluorescein is not a significant dissociation or cleavage product of the conjugate (with possible exceptions in organs which excrete it, namely the liver and kidneys). Although label shifting cannot be critically evaluated from the nature of these experiments, they do imply that if label shifts do occur, they are predominantly within the protein family, since all label behavior observed *in vivo* is compatible with substances of colloidal dimensions.

Fluorescein-labeled plasma proteins are preeminently qualified within a limited range of application. Whenever the visualization of plasma proteins is of decided advantage or the use of isotope labeling is impractical, fluorescein labeling may offer the best solution. No other method of labeling, without considerably more denaturation, is suitable for direct observations of the transcapillary passage of plasma proteins microscopically. Similarly, histological localization by fluorescence microscopy, while moderately complex at present, offers rapid inspection and seemingly better resolution than obtainable with radioisotope labels.

SUMMARY

Fluorescein–bovine albumin conjugates have been prepared and found not to differ appreciably in size, shape, and homogeneity from the precursor, bovine serum albumin. Fluorescein has also been conjugated to rat plasma proteins.

Their disappearance rates from the circulation of rats correspond with those obtained from the use of isotope labeling.

Their sites of localization in rat tissues were shown to be in the cytoplasm but not in the nuclei of Kupffer cells, fixed macrophages, granulocytes, and proximal renal tubules. Adsorption to endothelium was a characteristic finding.

Extracellular localizations were predominantly in the lumina of blood vessels and proximal renal tubules (but never in the lumina of collecting tubules), and the interstitial fluid of skeletal and cardiac muscle (but not that of glandular organs such as the adrenals, liver, and spleen).

BAC absorption from the skin of rabbits requires days whereas sodium fluorescein absorption is measured in hours, attesting to the persistence of the colloidal state of BAC *in vivo*.

Fluorescein conjugates have been used to visualize the transcapillary passage of circulating proteins in the mesenteric circulation of frogs and rats by direct microscopic observation and found to diffuse slowly in the manner predicted for plasma proteins.

The normal cutaneous vessels of the rat are impermeable in the gross to the labeled proteins; second degree burn promptly increases the permeability of these vessels rendering the presence of the label detectable in the gross in the skin.

The process of labeling does not render guinea pig albumin antigenic, although slight antigenicity results from labeling whole plasma protein.

It is believed that sufficient biological evidence is presented to support the conclusion that fluorescein-conjugated plasma proteins, particularly albumin, behave *in vivo* like their native precursors.

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BIBLIOGRAPHY

1. Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E., *J. Immunol.*, 1942, **45,** 159.
2. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, **91,** 1.
3. Sterling, K., *J. Clin. Inv.*, 1951, **30,** 1228.
4. Longsworth, L. G., *Chem. Rev.*, 1942, **30,** 323.
5. Philpot, J. S. L., *Nature*, 1938, **141,** 283.
6. Svensson, H., *Kolloid-Z.*, 1940, **90,** 141.
7. Hess, E. L., and Cobure, A., *J. Gen. Physiol.*, 1950, **33,** 511.
8. Svedberg, T., and Pedersen, K. O., The Ultracentrifuge, Oxford University Press, 1940, 38.
9. Abdou, I. A., and Tarver, H., *J. Biol. Chem.*, 1951, **190,** 769.
10. Adamstone, F. B., and Taylor, A. B., *Stain Technol.*, 1949, **23,** 109.

11. Gersh, I., *Bull. Internat. Assn. Med. Mus.*, 1948, **28,** 179.
12. Marshall, J. M., *J. Exp. Med.*, 1951, **94,** 21.
13. Smetana, H., *Am. J. Path.*, 1947, **23,** 255.
14. Coons, A. H., Leduc, E. H., and Kaplan, M. H., *J. Exp. Med.*, 1951, **93,** 173.
15. Coons, A. H., personal communication.
16. Kruse, H., and McMaster, P. D., *J. Exp. Med.*, 1949, **90,** 425.
17. Watson, B. K., *Proc. Soc. Exp. Biol. and Med.*, 1952, **79,** 222.
18. Hopkins, S. J., and Wormall, A., *Biochem. J.*, 1933, **27,** 740.

EXPLANATION OF PLATES

PLATE 3

All photomicrographs on this and subsequent plates taken on Eastman Kodak spectrographic film, type J (green sensitive emulsion), used with a fluorescence microscope equipped with a Bausch and Lomb cardioid condenser, 10 × ocular, and 4 mm. achromatic objective, except where otherwise noted.

FIG. 1. Liver of rat fixed in formalin. Sacrificed 12 hours following a single intravenous injection of homologous fluorescein plasma protein conjugate. Sectioned by CO_2 freezing. Conjugate-laden Kupffer cells stand out as bright fluorescent specks. × 59.

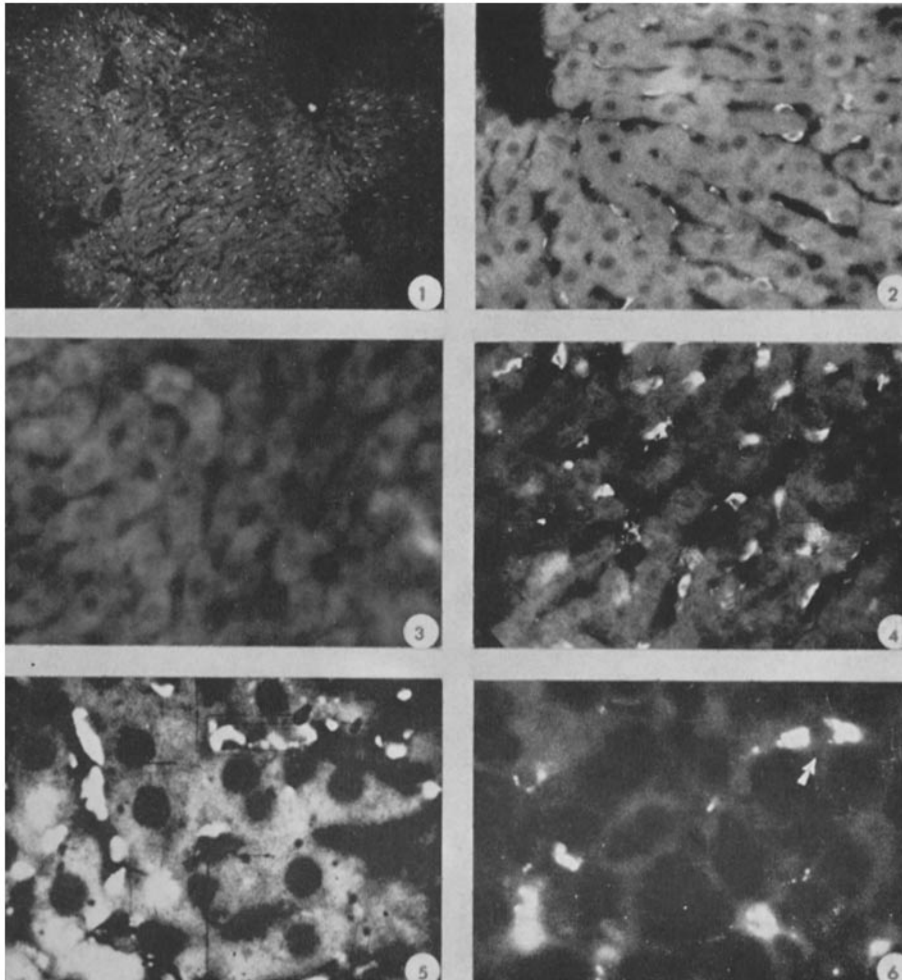
FIG. 2. Liver of rat prepared by freezing-dehydration method. Sacrificed 19 hours following a single intravenous injection of homologous fluorescein plasma protein conjugate. Conjugate-bearing Kupffer cells line the sinusoids. The cytoplasm of the hepatic cells shows strong autofluorescence characteristic of this method of preparation. × 296.

FIG. 3. Liver of rat fixed in formalin. Sacrificed 12 hours following a single intravenous injection of bovine albumin. Sectioned by CO_2 freezing. Kupffer cells are not visible in the absence of conjugate. Same exposure as Fig. 4. × 296.

FIG. 4. Liver of rat fixed in formalin. Sacrificed 12 hours after a single injection of BAC. Sectioned by CO_2 freezing. Kupffer cells now plainly visible. × 296.

FIG. 5. Enlarged detail from the section of tissue used for Fig. 2. Overexposed to emphasize the absence of fluorescence (label) in the hepatic nuclei. × 459.

FIG. 6 Liver of rat fixed in formalin. Sacrificed 16 hours after a single injection of BAC. Sectioned by CO_2 freezing. Incorporation of the label in the cytoplasm outlines the non-fluorescent nucleus of the Kupffer cell (arrow). × 459.



(Schiller *et al.*: Fluorescein-conjugated bovine albumin)

PLATE 4

FIG. 7. Kidney cortex of rat prepared by freezing-dehydration method. Sacrificed 19 hours following a single intravenous injection of homologous fluorescein plasma protein conjugate. Selective localization is evident from the appearance of bright clumps of tubules adjacent to others containing little or no label. Arrows indicate presence of glomeruli. × 59.

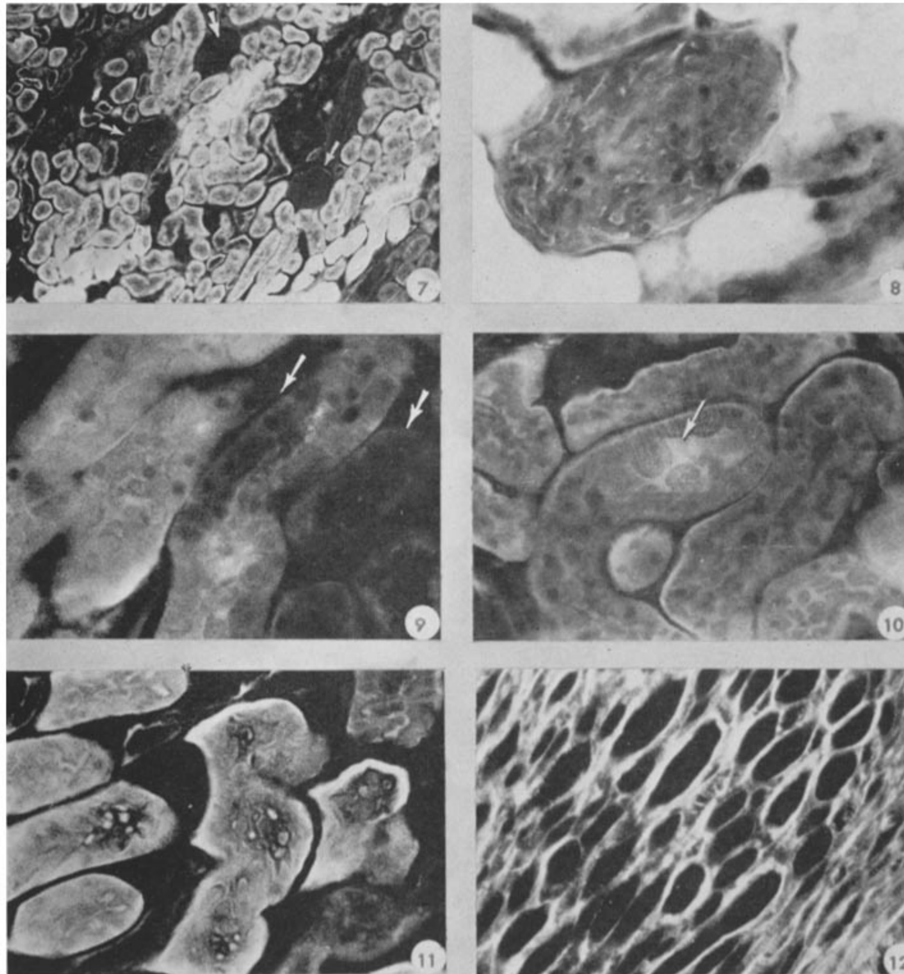
FIG. 8. Kidney cortex of rat prepared as above. Sacrificed 12 hours following a single intravenous injection of BAC. Delicate outlines of glomerular capillaries revealed by adsorbed conjugate. (Overexposed.) × 296.

FIG. 9. Kidney cortex. Enlarged detail from the section of tissue used for Fig. 7. Selective absorption of label indicated by its absence from some cells within a tubule and from adjacent tubules (arrows). × 424.

FIG. 10. Kidney cortex. Enlarged detail from the section of tissue used for Fig. 7. Label is present in relatively high concentration within the lumen of a tubule (arrow). × 424.

FIG. 11. Kidney cortex. Enlarged detail from the section of tissue used for Fig. 8. Presence of label within lumina of tubules is evident (Bausch and Lomb UV reflecting condenser). × 340.

FIG. 12. Kidney medulla from the same slide used for Fig. 11. No evidence of label within the lumina of collecting tubules. Brightness of parenchyma largely due to autofluorescence. × 296.



(Schiller *et al.*: Fluorescein-conjugated bovine albumin)

Plate 5

Fig. 13. Heart, cross-section through endocardium. Same animal and method of tissue preparation as used for Figs. 8 and 12. Label localized in interstitial fluid between muscle bundles. × 254.

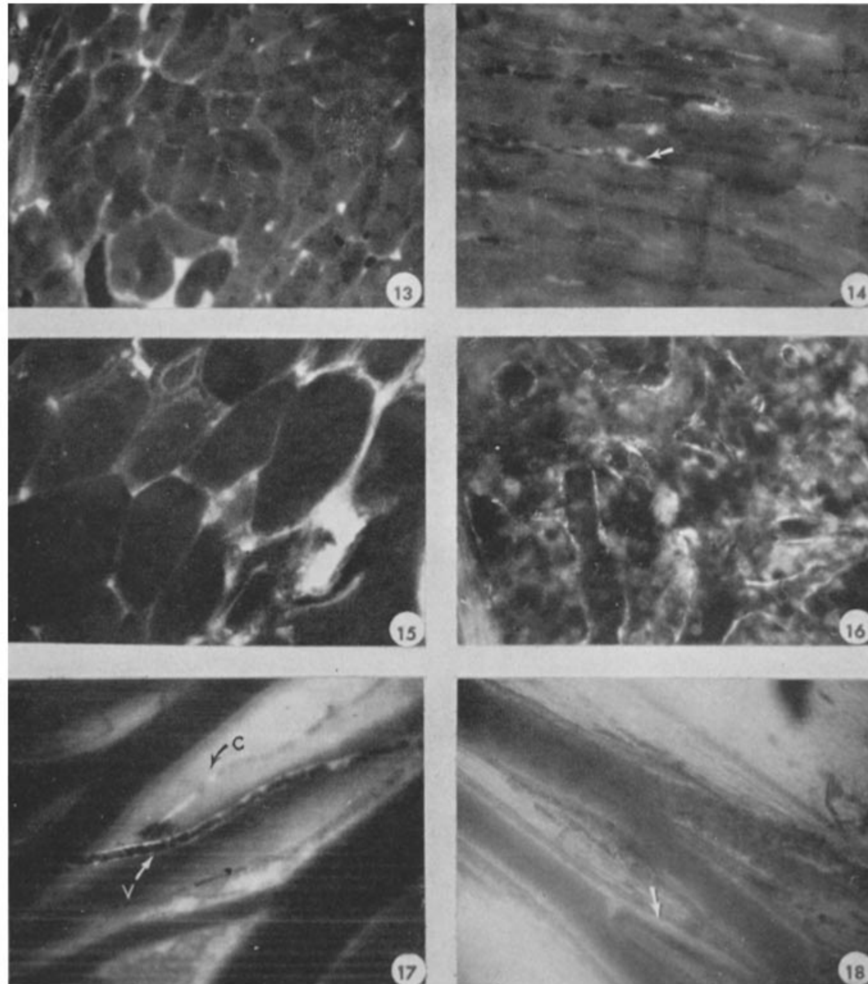
Fig. 14. Heart, longitudinal section, of rat prepared by formalin fixation. Sacrificed 19 hours following a single intravenous injection of homologous fluroescein plasma protein conjugate. Label faintly visible in interstitial fluid between muscle bundles, and in cytoplasm of fibroblast (?) (arrow). × 336.

Fig. 15. Skeletal muscle, cross-section. Same animal and method of tissue preparation as used for Figs. 8 and 12. Label localized in interstitial fluid surrounding muscle fasicules (Bausch and Lomb UV reflecting objective and condenser). × 418.

Fig. 16. Spleen of rat prepared by formalin fixation. Sacrificed 14 hours after a single intravenous injection of BAC. Adsorption of labeled protein on endothelium traces delicate outline of sinus arborization. Bright areas in pulp are not due to fluorescence of label. × 295.

Fig. 17. Mesentery of frog *in vivo* 97 minutes following an intracardiac injection of BAC. Red cells cast shadows in the conjugate-filled lumen of a capillary (*C*). Label-visualized "plasma sleeve" and central core of red cells in venule (*V*). (E. K. Super XX film, 1/5 second.) × 59.

Fig. 18. Mesentery of frog *in vivo* 190 minutes following intracardiac injection of BAC. Label-visualized plasma sleeve in large veins. Plasma "skimming" evident at bifurcation (arrow). (E. K. Super XX film, 10 seconds.) × 59.



(Schiller *et al.*: Fluorescein-conjugated bovine albumin)