CONJUGATED FLUORESCEIN FOR FUNDUS PHOTOGRAPHY*†

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FLUORESCEIN has been found to be of value in ophthalmology as an intravital stain for studying fundus pathology, and the dye has always been injected as the aqueous solution. MacLean and Maumenee (1960) used a slit lamp to observe fluorescence in a haemangioma of the choroid after intravenous injection of 2 ml. of a 5 per cent. solution, and later used a slow injection (over 2 minutes) of 10 ml. of the 5 per cent. solution to obtain a satisfactory view of a similar tumour by indirect ophthalmoscopy. Other workers have obtained photographic records of fundus fluorescence after intravenous injection of 5 ml. (Dolley, Hodge, and Engel, 1962; Novotny and Alvis, 1961) or 10 ml. of a 5 per cent. solution (Linhort, McIntosh, Heyman, and Host, 1964), while others have used 4 ml. (Rubinstein and Paton, 1966) or 5 ml. of a 10 per cent. solution (Miller, Sanders, and ffytche, 1965). Oosterhuis and Lammens (1965) used 3 ml. of a 20 per cent. solution to increase fluorescence and enable records to be made on fine-grain film. No other alternative doses have been recorded.

Aqueous fluorescein does not appear to conjugate readily with the plasma proteins, and disappears rapidly from the vessels after intravenous injection. Dollery and others (1962) noted that aqueous fluorescein diffuses into a space four times the plasma volume within 10 minutes of injection. He later showed that the green colour emitted by fluorescein is absorbed by the red haemoglobin so that the ratio of the concentrations of the dye in blood and saline which were required to give the same density of photographic image was 20 to 1.

Conjugation of fluorescein with a large molecule such as albumin would contain the fluorescein within the vascular system and indicate areas of damaged capillary permeability, while conjugates with various sizes of dextran might again give different patterns. The mechanism of conjugation is a reaction between sulphonic acid groups in fluorescein isothiocyanate with free amino and carboxyl groups in the protein chain—the sulphonamide link is particularly stable. There is an optimum ratio of fluorescein to protein molecule for maximum fluorescence, and beyond this an increase in the number of fluorescein molecules does not produce a corresponding increase in the fluorescence. Schiller, Schayer, and Hess (1953) conducted a series of experiments to find whether conjugation of fluorescein with serum albumin could be used for studies of the permeability of capillaries to circulating protein. After many tests they concluded that the only difference between the conjugate

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and naturally occurring albumin was a change in iso-electric point. It is theoretically possible for a conjugate to act as a hapten and stimulate antibody production, but this effect appears to be slight. There was no evidence of anaphylaxis when only albumin was labelled, but some reactions occurred if whole serum was used. These workers then investigated the results of intravenous injection of fluorescein-conjugated albumin in various animals. Among other studies they produced a cutaneous thermal burn on the rat abdomen 20 hours after intravenous injection of the conjugate, using 25 mg./100 g. body weight. One hour later, examination under ultra-violet light showed that fluorescence was localized to the site of the burn. Their studies of frog mesentery by fluorescence microscopy showed that vessels were visible for 15 to 30 minutes after injection; then diffusion began, but after 2 hours there was still a blood/tissue gradient. They showed that the conjugate disappeared from the circulation at the same rate as isotopically-labelled plasma protein. Laurence (1952) showed that there is a rapid fall in the concentration of conjugated albumin for 48 hours after intravenous injection because of distribution in the tissues, followed by a slower fall with a half-life of 5 days. He found no evidence of immediate or delayed harmful effects from protein/fluorescein conjugates after intravenous injection in rabbits, rats, or guinea-pigs. This work gives support to the possibility that a conjugated fluorescein might be of use in ophthalmology, particularly in differentiating exudative lesions from other fundus disturbances. The present experimental work was carried out on rabbits to investigate this. It was thought that it might be a valuable aid for study and diagnosis of ocular conditions such as diabetic retinopathy, central serous retinopathy, and papilloedema if an alternative to the small molecule aqueous fluorescein was available which would give a different pattern of stain.

**Method and Results**

The amounts used were selected to obtain as much conjugation as possible. 280 mg. freeze-dried rabbit albumin in phosphate buffer at pH 7·4 was added to 33 mg. fluorescein isothiocyanate dissolved in the same buffer. The mixture was allowed to stand overnight to allow ample time for reaction. It was then passed down a Sephadex G25 column to separate the free dye. The conjugate was the fastest band and was collected after ultrafiltration. The final solution contained 280 mg. conjugate in 14 ml. Another solution containing 280 mg. in 4 ml. was also prepared. Two dextrans with molecular weights of 10,000 and 250,000 were also conjugated in a similar way. 1 g. dextran was dissolved in 10 ml. buffer and offered 10 mg. fluorescein isothiocyanate. The mixture was left to stand for 3 days to allow conjugation to take place. This was considered to be necessary because the hydroxyl group of dextran is not as reactive as the amino group of protein. A large amount of free dye was removed by precipitation with alcohol. This gave 1 g. conjugate in 12 ml. An attempt was made to produce more efficient labelling by reducing the volume of buffer, but only a slight improvement was obtained.

Spots of the conjugates were put onto white filter paper for examination. In daylight the strongest albumin conjugate has the same yellow colour as aqueous fluorescein of about 1 per cent. concentration. Both dextrans accepted only enough fluorescein to give a pale straw-coloured spot, the smaller molecule dextran being the weaker of the two. Under ultra-violet light spots of both the albumin conjugates fluoresced well, but fluorescence could be obtained only from the bulk solutions of the dextrans. In the light of the fundus camera, however, with the blue Wratten gelatine filter No. 47 in position, the albumins
were only moderately fluorescent. The dextran were completely quenched and were not used in any further experiments.

The first experimental procedure was to inject 2 ml. of the albumin conjugate into a 3 kg. rabbit by the intravenous route. Blood samples were collected 5 minutes, 2 hours, and 4 hours later. One drop from each was placed on a microscope slide with a cover-slip. These were then examined in ultra-violet light and all specimens showed a similar degree of fluorescence, indicating that the conjugate was remaining in the circulation and not being removed by the reticular system.

A brown rabbit was then anaesthetized and a small burn produced by trans-scleral diathermy through a conjunctival incision. A suitable burn was one in which there was a barely visible grey area of fundus disturbance, which could be produced by a current of 40mA applied for 4 seconds. The intention was to damage the choroidal capillaries without destroying the circulation, and so produce an exudative lesion. This appearance could be easily distinguished from a more severe burn producing coagulation of the tissues with choroidal haemorrhage. This procedure was immediately followed by the intravenous injection of 2 ml. conjugate (50 mg.), and 2 hours later the animal was again anaesthetized and the lesion viewed through the Zeiss fundus camera. The lesion could easily be located with the white light, but with the blue filter in place there was no fluorescence. The lesion was re-examined at intervals during the next 1½ hours but there was no visible fluorescence in the lesion. The anaesthetic was continued and a test injection of 0·4 ml. of 10 per cent. aqueous fluorescein was given intravenously. The lesion immediately fluoresced brightly, indicating an intact circulation. The fluorescence persisted for several minutes, suggesting capillary damage with exudation of dye. The experiment was then repeated using another rabbit and injecting 140 mg. conjugate in 2 ml. Although this was almost three times the previous dose there was still no fluorescence in the fundus lesion 2 hours after injection. It was considered that it would be of no value to increase the dose of the conjugate as the investigation was planned to discover if there could be a clinical application—an injection of 2 ml. in a rabbit is equivalent to about 50 ml. in a human being on a similar volume/weight basis.

Discussion

These negative results are reported because it would be a diagnostic aid to be able to obtain different patterns of stain in the fundus by using different fluorescent molecules. Although other workers had reported differences in the intensity of emission from aqueous and conjugated fluorescein it does not appear to have been tested previously in ophthalmological use. Schiller and others (1953), in their studies, found comparable emission from the free and conjugated dye in ultra-violet light, and there were no new peaks in the absorption spectrum. However, they found differences when the fluorescence was excited by the visible spectrum. At the peak excitation wavelength of 490–495 mμ the albumin conjugate showed about 50 per cent. of the fluorescence by the aqueous solution because of reduced absorption of light. Laurence (1952) found an even weaker intensity of fluorescence by the conjugate relative to the free dye. He noted that many fluorescent dyes were quenched by conjugation to protein. In spite of these reports it was considered possible that 140 mg. of the conjugate retained in the volume of the blood stream would fluoresce as strongly as a 40 mg. injection of the aqueous solution.
Summary

An attempt was made to determine whether conjugated fluorescein would give a different pattern of stain to the aqueous dye after intravenous injection. An experimental fundus lesion was produced which did not fluoresce after injection of an albumin conjugate but fluoresced well after the aqueous solution was injected. It appears that this conjugate cannot be adequately excited by the filtered blue light used for fluorescein fundus photography.

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