Studies in Rhodopsin

4. PREPARATION OF RHODOPSIN

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The history of visual pigments has been reviewed on numerous occasions—see Collins & Morton (1950) and Wald (1951). In recent years the methods used in preparing solutions of rhodopsin have involved at least partial separation of rod outer segments. If retinas are shaken vigorously in saline or other suitable suspending medium the rod outer segments become detached. Various methods have been used for separating these 'rods' from the remainder of the retinal suspension. Lythgoe (1937) poured the suspension through fine wire gauze and showed that the rods, pigment granules and a few isolated cells passed through, but that the main mass of nuclear and fibrous material was held back. Krause & Sidwell (1938) centrifuged the retinal suspension and found that the rods formed a layer on top of the other retinal fragments which could then be scraped off. Saito (1938) used 40–45 g. sucrose/100 ml. water as the suspending medium and found that on centrifuging, the rods remained in suspension. The rods could be thrown down when diluted with sufficient saline. The method has been used by Collins & Morton (1950), by Bliss (1950) and by Wald (1951). In addition, Wald (1949) has often simply scraped the rods off the retina with a fine spatula or forceps.

The purity of solutions of rhodopsin prepared from rods isolated by any one of the methods described above varies greatly. With frog retinas it is easier to obtain pure solutions than with cattle retinas. It was the purpose of the present work to investigate more fully the reasons for these differences and to develop improved techniques applicable to cattle retinas which can be obtained from abattoirs in large numbers. Comparatively large amounts of cattle rod outer segments would greatly facilitate future work.

EXPERIMENTAL

Solutions

Potash alum $K_2SO_4.Al_2(SO_4)_3.24H_2O$, 4% (w/v) in water.

Digitonin solution. 1 or 2% (w/v) in water; prepared by heating the mixture of digitonin and water to boiling until solution occurs and then quickly cooling.

Buffer solution, pH 9·3. $Na_2B_4O_7.10H_2O$, 19 g./l.

Formaldehyde (neutral). A 40% (w/v) solution of formaldehyde was shaken with $MgCO_3$, allowed to stand overnight and filtered.

Animals

Eyes were obtained from freshly killed cattle and transported from the local abattoir to the laboratory in closed tins. The dissection, carried out in red light, has been described by Collins & Morton (1950).

The frogs used were *Rana esculenta.*

Absorption measurements

All measurements were made with a Beckman photoelectric spectrophotometer. A 1 cm. cell was used. The compensating cell contained a solution of digitonin and buffer equivalent in strength to that used for the rhodopsin solutions. The cells were filled in the dark room (using red light), placed in the cell holder and covered with a black cloth. They were then carried to the Beckman and placed in position in the light-tight cell space. Although rhodopsin solutions are sensitive to light no noticeable photodecomposition took place during measurements.

Criteria of purity

The spectroscopic purity of rhodopsin solutions has been assessed as follows. The ratio of the extinction at wavelength λ to that at $\lambda_{max.}$ (about 500 mμ.) has been designated P_λ. It is found that in the presence of absorbing impurities P_λ is higher than that for pure rhodopsin. Hence as the degree of purity increases P_λ will tend to a constant low value. Usually P_λ has been measured at 400 mμ., a minimum in the rhodopsin curve (cf. Collins & Morton, 1950). The lowest value so far obtained for P_{400} is about 0·25. As rhodopsin solutions have an absorption maximum near 275 mμ. due to the presence of protein (tyrosine and tryptophan) and also some nucleotide absorption near 260 mμ. (Collins, Love & Morton, 1952) it has been found useful to record P_{275} in some cases.

RESULTS

Preliminary investigations

Separation of rods by scraping. Wald (1949) separated rods by carefully scraping the surface of the retina with a fine spatula. This method was investigated.

Cattle retinas were spread out flat on a piece of filter paper with the rods uppermost. The surface was then gently stroked with a coarse hair brush, a spatula having been found useless, and the brush shaken at frequent intervals in saline solution. The saline suspension, when examined microscopically, was seen to contain large fragments of tissue, rods and blood cells. In another experiment a fine camel-hair brush was used and microscopical examination revealed a suspension of rods and blood cells only. In both

cases a sugar separation (see next section) was subsequently carried out, the rods hardened in alum and finally extracted with aqueous digitonin solution. The solutions yielded values of P_{400} of 0·53 and 0·68 respectively.

These results and the extra time required made this method unsuitable for cattle retinas.

Crude rod suspensions. These can be obtained by shaking a number of retinas in saline or sucrose solutions and pouring through a fine wire gauze (60-mesh). The resulting suspension contains most of the rods but is heavily contaminated with other tissue fragments. Lythgoe (1937) pointed out that frog retinas do not fragment during shaking, apart from releasing the rod outer segments, but cattle retinas as a whole appear to break up into small pieces. This we confirmed. Frog and cattle retinas were shaken vigorously in saline. The suspension of frog 'rods' was almost uncontaminated by other tissue fragments, but the suspension of cattle rods contained many blood cells and a high proportion of unwanted tissue and melanin pigment granules. The frog rod outer segments were much the larger.

Separation of rods by Saito's method

Saito's method, as modified by Collins & Morton (1950), was used at first. However, the purity of cattle rhodopsin solutions obtained by this method was very variable and the need for improvement soon became apparent.

The original method was as follows. The cattle retinas, dissected in red light, were shaken vigorously in 1·32 M-sucrose for 30 sec. using 15 ml. sucrose solution per twenty-four retinas, and poured through the fine wire gauze. The resulting suspension was centrifuged at 1600 *g* for 15 min. At the end of that time it could be seen that the fibrous part of the retina, blood cells and melanin were thrown to the bottom of the tube, while the rods remained suspended in the supernatant. This was decanted, diluted with 2–3 vol. of 0·9% (w/v) saline and centrifuged until clear. The brilliant scarlet precipitate was hardened in 4% (w/v) alum solution for 1 hr. The rods were then centrifuged down and washed once with saline, and finally extracted for 1 hr. with 1 ml. of 1% (w/v) digitonin solution and centrifuged. The clear supernatant was mixed with 1 ml. of buffer solution (pH 9·3), recentrifuged, and the absorption spectrum measured.

The following variables were investigated:

(i) The effect of pH on the digitonin extraction. The least amount of contaminating impurities were extracted at an acid pH, but a fine precipitate tended to form which could not be centrifuged down. Alkaline extracts were very impure. Hence a neutral extractant was employed.

(ii) The concentration of sugar seemed unimportant between about 1·0 and 1·6 M, but below 0·88 M the rods sank to the bottom and formed a layer on the debris, while above about 1·6 M the whole brei floated.

(iii) Re-extraction of the retinal debris with 1·32 M-sucrose yielded a less pure fraction. More rods were obtained, but with four successive 'sugar extractions' the purity deteriorated thus: P_{400} = 0·446, 0·53, 0·733 and 0·858.

(iv) The duration of the alum treatment is apparently unimportant provided it exceeds 30 min. If the rods are left for periods greater than 24 hr. at room temperature there is a slow loss of rhodopsin.

(v) The extraction by means of digitonin solution is nearly complete in 1 hr., but thereafter increasing amounts of impurities go into solution.

Attempts to remove impurities from rods. Rods separated by Saito's method were extracted with an alkaline buffer (pH 9·3) followed by an acid one (pH 4·1). The rods were then hardened in alum and extracted as usual; P_{400} = 0·477. In other experiments the rods were extracted with 0·1% Dispersol A (an Imperial Chemical Industries Ltd. detergent); untreated rods gave a value of P_{400} = 0·783, whilst after treatment P_{400} became 0·565. Further experiments showed that only very heavily contaminated rod preparations were improved.

The following organic solvents were tried: phenol, acetone, amyl alcohol, pyridine, *cyclo*hexane, ethyl acetate and ethyl butyrate. All except the last bleached the rhodopsin immediately—ethyl butyrate did so in about 24 hr.

It was noticed that rods spun down after having been separated were contaminated with many black particles. Attempts were made to separate these small particles using a fine spatula. A typical experiment was as follows: the rods were divided into two portions: (1) was used as a control and (2) was separated into (2*a*) rods relatively free from black particles and (2*b*) rods contaminated with black particles. The values of P_{400} were respectively 0·282, 0·312 and 0·362. In another experiment the 'clean' rods gave P_{400} = 0·30 and contaminated rods gave P_{400} = 0·37. The conclusion to be drawn from this experiment was that even the 'cleaned' rods gave less pure solutions than those obtained from untreated rods.

Treatment with formaldehyde. Although ordinary formaldehyde solutions (40%, w/v) at once destroyed rhodopsin it was found that a neutralized solution (i.e. left to stand with magnesium carbonate overnight) did not. It was found that solutions of rhodopsin prepared from rods treated with formaldehyde had a value of P_{275} lower than had been obtained previously and that 10 min. in formol was the optimum time of treatment (Tables 1 and 2). Good results were obtained by combining formol treatment with differential centrifugation (see below) in winter and spring, but the results in summer were unsatisfactory (Table 2). The purest cattle rhodopsin solution obtained by this method had P_{400} = 0·236, P_{275} = 2·05 at pH 9·3 (Fig. 2). Later experiments have shown that it is better to treat with alum before the formol treatment.

Attempts to remove impurities from rhodopsin solutions. Various attempts were made to precipitate the rhodopsin from its digitonin solution in the hope that impurities would be left in solution. Acetone was tried first; this solvent destroys rhodopsin at room temperature but not at 0°. However, it was found that all the protein present was precipitated and that no purification resulted. Addition of acetone in steps might be more successful if combined with facilities for low temperature centrifugation.

Sodium sulphate and magnesium sulphate and sodium chloride were next tried, but did not result in any improvement when the precipitated rhodopsin was redissolved. Ammonium sulphate was more successful.

Table 1. *Effect of duration of treatment of rods with formaldehyde on purity of rhodopsin*

(Cattle rod outer segments were treated with neutralized formaldehyde solution for various periods and then treated with alum and extracted with digitonin solution as usual. The data refers to the absorption spectra of the resulting solutions.)

Time in formol (min.)	P_{400}	P_{275}	$E_{500m\mu.}$
	First experiment		
0	0·322	3·3	0·251
10	0·318	2·45	0·277
20	0·322	2·55	0·192
40	0·350	2·88	0·148
17 hr.	About half of the rhodopsin destroyed		
	Second experiment		
0	0·330	3·64	—
5	0·295	2·45	—
10	0·295	2·11	—
15	0·300	2·18	—

original solution. The second fraction contained a little rhodopsin of low optical purity. Fractions 3 and 4 contained no rhodopsin, but had maxima near 270 mμ.

These results showed that rhodopsin solutions contained irrelevant material absorbing at 275 mμ. and that some of this impurity could be removed by fractional precipitation with ammonium sulphate. Better results were obtained later by another method.

Modifications of Saito's method

Differential centrifugation. Rods from twenty-four ox eyes were prepared by Saito's method. The sugar suspension was diluted with 3 vol. of saline and centrifuged for 2 min. only at 1200 *g*. The precipitate was treated with alum and the supernatant cleared by centrifuging for 15 min. at 1800 *g*, and this second precipitate was also treated with alum. When the precipitates were extracted, the values of P_{400} were 0·28 and 0·38 respectively. The value of P_{275} for the first solution was 2·29.

It was found that for this method to be effective the eyes had to be dissected and extracted very soon after death. Another disadvantage was the low yield—less than 20 % of the total rhodopsin/retina.

Table 2. *The effects of treating rod outer segments with formaldehyde solution on purity and yield of rhodopsin*

(Cattle rod outer segments were treated with neutralized formaldehyde solution for 10 min., then with alum for 1 hr. and extracted as usual with digitonin solution. The data refer to the absorption spectra of the resulting solutions.)

Solutions prepared in the winter

	Formol treatment			No formol treatment		
	P_{400}	Yield*	P_{275}	P_{400}	Yield*	P_{275}
Mean	0·303	0·056	2·80	0·293	0·066	3·84
Standard error	0·0089	0·0075	0·15	0·0096	0·0064	0·33
No. of samples	19	18	19	13	12	14
Range	0·236–0·39	0·02–0·14	2·05–4·9	0·217–0·34	0·03–0·10	2·8–7·2

Solutions prepared in the summer

	Formol treatment		No formol treatment	
	P_{400}	P_{275}	P_{400}	P_{275}
Mean	0·515	6·9	0·324	4·1
Standard error	0·0605	0·87	0·017	0·29
No. of samples	11	10	11	9
Range	0·26–0·93	2·9–11·3	0·27–0·44	2·95–5·9

* $\text{Yield} = \dfrac{(\text{Extinction at 500 m}\mu\text{. due to rhodopsin}) \times (\text{vol. of soln.})}{(\text{No. of retinas})}$.

Rods from fifty cattle eyes were extracted three times with 1 ml. of 0·5 % digitonin solution and the combined extracts were mixed with an equal volume of buffer, pH 4·5. The resulting solution had $P_{400} = 0·575$, $P_{275} = 7·7$. On adding increasing amounts of solid ammonium sulphate four fractions were obtained. The first contained most of the rhodopsin, P_{275} and P_{400} both being lower than in the

The sugar gradient method. The combination of differential centrifugation and extreme freshness of the eyes in the Saito method produced many solutions of cattle rhodopsin purer than had ever been obtained before, but in view of the low yield and the difficulty experienced in obtaining sufficiently fresh eyes a new modification was developed and is described below.

Retinas are shaken vigorously with about half their volume of 0·9 % (w/v) saline in order to detach the rod outer segments. The mixture is stirred into a piece of 60-mesh brass gauze shaped in the form of a hollow until all the liquid has passed through, whereupon the retinal debris is transferred to a glass tube and shaken again with saline. This treatment is done four times, after which the retinal fibrous mass becomes considerably reduced in bulk and is usually colourless. The resulting suspension contains tissue fragments, blood, melanin and rods. It is transferred to a centrifuge tube, which should not be more than three-fifths filled by it. Saturated sucrose solution—greater than 2·0 M—is now carefully poured down the side of the tube so as to form a lower layer of about half the volume of the saline

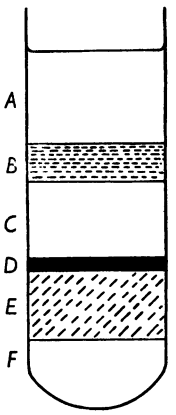

Fig. 1. This diagram shows the disposition of the various tissue fragments after being centrifuged in sucrose solution in which a density gradient had been established as described in the text (p. 295). *A*, pale opalescent solution containing some protein; *B*, dense, deep scarlet, layer of rod outer segments; *C*, faintly pink opalescent layer containing a few rod outer segments and some small tissue fragments; *D*, dense, dark red, layer containing mostly red blood cells; *E*, densely packed layer, containing the greater bulk of the disintegrated retina as well as melanin granules; *F*, colourless, almost saturated, sucrose.

suspension. The next part of the process requires practice. A flat-ended glass rod is used, and with it the saline-sugar interface is stirred. The object is to produce a gradient of concentrations of sucrose down the tube, ranging from zero, i.e. saline only, at the top, through gradually increasing concentrations, to saturated sucrose at the bottom. The tube is then centrifuged at 1800 *g* for 15–20 min. On removing the tube from the centrifuge, all the components of the suspension are found to have accumulated in their own pycnotic level.

Fig. 1 shows a diagram of an actual separation. The various zones are sucked off using a tube drawn out at the end and turned up so as to resemble a crochet hook. The rods are diluted with 2–3 vol. of saline and centrifuged down.

It was found that sucrose solutions in a series of strengths from 0·76 to 1·17 M at 0·06 intervals could, by careful pipetting, be placed one on top of another in a centrifuge tube without mixing appreciably. The strongest ones were, of course, on the bottom, and the boundaries between the solutions could be clearly seen. Their positions were then marked. The crude saline suspension of rods was pipetted on top of them all and the whole centrifuged. It was found that cattle rods are isopycnotic with 0·88 (± 0·15) M-sucrose, while the blood cells, etc., come to rest in sucrose of greater than 1·0 M. This being so, it was hoped that, by making a series with a deep 0·94 M layer, the rods could be separated a long way from the blood cells. The idea was eventually abandoned, however, since once the technique had been learned the stirring method was far less tedious. The rods from frogs appear to be denser than cattle rods.

Table 3. *Variations in the purity and yield of rhodopsin solutions from rod outer segments prepared by the sugar gradient method*

(Cattle rod outer segments were separated by flotation in sucrose solution with a density gradient. The isolated rods were treated with alum and extracted with digitonin solution. The data refer to the absorption spectra of the resulting solution.)

	P_{400}	P_{275}	Yield*
Mean	0·293	3·83	0·212
Standard error	0·0061	0·13	0·038
No. of samples	16	14	11
Range	0·26–0·34	3·1–4·5	0·05–0·49

* Yield $= \frac{(\text{Extinction at } 500\ \text{m}\mu\text{. due to rhodopsin}) \times (\text{vol. of soln.})}{(\text{No. of retinas})}$.

If the rod preparation was not very pure, then a repetition of the gradient process effected an improvement, but further treatments only worsened the preparation, due, no doubt, to excessive handling of the rods. In any second purification, the top layer was usually water-clear, but a little retinal tissue could be seen under the rod 'band'.

Table 3 shows that the yield of rhodopsin per retina is greatly improved and that the values of P_{400} indicate fairly good purity.

For these procedures it was unnecessary to use extremely fresh eyes. On one occasion 130 eyes were treated in one day and, although the time taken for dissection and treatment was considerable, the value of P_{400} for an extract of some of the resulting rods was 0·30.

Microscopic examination of rod suspensions in the course of various treatments

No staining or fixing was used.

(*a*) Sugar separation. (i) Crude rod suspension. Rods were mostly intact with many red blood cells and other, not easily distinguished, tissue fragments present. (ii) After a sugar separation. Some rods were deformed, but the majority were unchanged. Very few other tissue fragments were present. (iii) After another sugar separation. Very few intact rods remained.

(*b*) Effect of time on a rod suspension in 0·9% (w/v) NaCl. (i) Fresh—rods remained intact. (ii) 0·5–1 hr.—some changes were noticed. (iii) 1–2 hr.—many deformed and broken rods were seen. (iv) 3 or more hr.—very few intact rods remained.

(*c*) Effect of various reagents on rod suspensions. (i) Formaldehyde (40% neutralized)—the rods were seen to curl up forming almost complete circles and sedimented readily. (ii) Digitonin solutions—the rods were broken up completely. (iii) Alum solutions (4%, w/v)—the rods were broken up completely and sedimented quickly. (iv) A solution containing 2% formaldehyde and 0·9% KCl—the same changes occurred as described under (*b*) but a little more slowly.

DISCUSSION AND CONCLUSIONS

Separation of rod outer segments

The separation of rod outer segments from the other tissue fragments present in the initial crude suspension depends on two factors, the size and the density of the rods. The rods are fairly large (about $3 \times 30\ \mu$.) and sediment rapidly in water even if the centrifugal force is as low as 600 *g*. In this respect they are comparable to red blood cells and nuclei. However, the rods differ from blood cells and nuclei in one important property, they are less dense and will float in 0·88 M-sucrose (sp.gr. = 1·114).

If these were all the facts then the methods described would separate rods from all tissue fragments except (*a*) particles isopycnotic with rods, and (*b*) particles so small that, at the centrifugal forces used (about 1800 *g*), they are removed too slowly from the rod zone. A second separation should enable the majority of small particles not isopycnotic with the rods to be eliminated. This presupposes that rod outer segments are stable. However, microscopic examination reveals quite clearly that they are not and that they fragment. This explains why a repetition of a sugar separation does not result in any great improvement. As the rods break up greater centrifugal forces would become necessary to effect a separation. This was not considered practical, and the writers prefer one sugar separation performed as quickly and as efficiently as possible. Subsequent treatments with alum and neutralized formaldehyde yield solutions which are very pure.

A recent study, using the electron microscope, of rod outer segments by Sjöstrand (1949) indicates that rods are composed of several thousand disks each about 3 mμ. thick. He records the fact that rods when broken up yield fragments which may contain a few disks or many hundreds. This is in good agreement with our own observations and reveals still more clearly the difficulties encountered in attempting to separate rod outer segments.

The effect of this fragmentation of rods will be manifested in two ways. The first will be the increased difficulty of effecting an efficient separation from other tissue fragments. The second and perhaps more serious will be the presence of submicroscopic particles (fragments containing only a few 'disks') which will not be centrifuged out after the digitonin extraction. This will make the final rhodopsin solution turbid.

Characteristics of pure (?) rhodopsin

In the absence of any precise chemical criterion of purity spectroscopic ones are used instead. A value of P_{400} below 0·24 to 0·26 has not yet been obtained (cf. Wald, 1951). Similarly, a value of P_{275} of less than 2·05 has not been obtained. The curve shown in Fig. 2 represents such an absorption curve and it is worth while to examine it in detail.

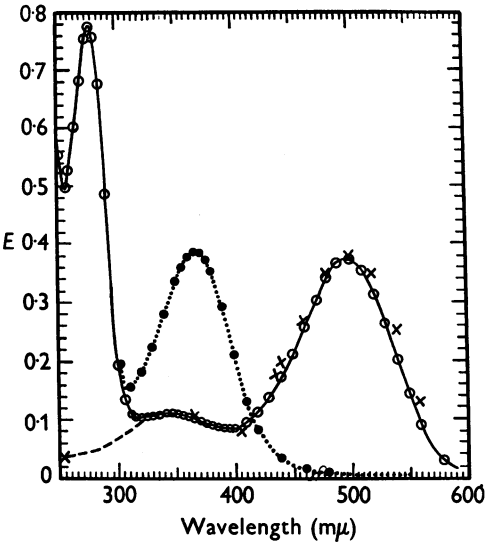

Fig. 2. –○—○–, absorption spectrum of a very pure solution of cattle rhodopsin, pH 9·2; × × ×, relative photochemical efficiencies. $[(\epsilon_\lambda \times \gamma_\lambda)/\epsilon_{500\ m\mu.} \times \gamma_{500\ m\mu.}$ multiplied by a factor so that the two curves correspond at 500 mμ.; after Schneider *et al.* (1939)]. ..●...●..., absorption spectrum of bleached solution (cf. Collins & Morton, 1950); ----, extrapolation indicating probable absorption of the rhodopsin chromophore below 320 mμ.

In Fig. 2 it is shown that a very close correspondence exists between the rhodopsin absorption curve, and the relative photochemical efficiency curve of Schneider, Goodeve & Lythgoe (1939). The last curve predicts a small peak near to 350 mμ. which is in fact found. It also predicts that at 254 mμ. the absorption of the rhodopsin chromophore should be only about 6% of that at 500 mμ. A plausible extrapolation for the absorption due to rhodopsin chromophore is shown in Fig. 2 by means of dashes. The difference between this and that actually found

will be due to the protein containing tyrosine and tryptophan and can be calculated as follows. If E_{280} represents the total extinction at 280 mμ. after subtracting the absorption due to the rhodopsin chromophore, and E_{290} is the corresponding value at 290 mμ. then

$$E_{280} = x + y,$$
$$E_{290} = 0{\cdot}448x + 0{\cdot}710y.$$

Here x = extinction at 280 mμ. due to tyrosine and y = extinction at 290 mμ. due to tryptophan, both at pH 9·2. (Collins & Morton, 1950, give similar equations; the difference is due to the fact that a purer sample of tryptophan was obtained for the present work.)

animal proteins is of the same order; casein (6·6 and 1·2), cattle fibrin (6·5 and 3·0), haemoglobin (3·2 and 1·3) serum albumin (4·8 and 0·5) and serum globulin (6·7 and 2·3).

It should be pointed out that the conclusion that the only absorbing materials present are the rhodopsin chromophore, tyrosine and tryptophan does not exclude small amounts of phenylalanine, nucleotides or other unidentified materials absorbing in the region 250–300 mμ.

It is not yet possible to say if the rhodopsin solutions now obtainable are 'pure', but they are in very close agreement with the results obtained by Wald and his co-workers (Wald, 1951). It seems likely that any contaminants present show no

Table 4. *Analysis of the ultraviolet absorption of a rhodopsin solution*

(Solution in 1% (w/v) aqueous digitonin and borate buffer, pH 9·2.)

λ (mμ.)	(1)	(2)	(3)	(4)	(5)	(6)	(7)
300	0·199	0·076	0·123	0·085	0·052	0·137	−0·014
290	0·491	0·058	0·433*	0·127	0·305	0·432	+0·001
285	0·681	0·053	0·628	0·214	0·370	0·584	+0·044
280	0·764	0·051	0·713*	0·283	0·430	0·713	0·000
275	0·753	0·047	0·706	0·297	0·415	0·712	−0·006
270	0·681	0·044	0·637	0·252	0·398	0·650	−0·013
265	0·600	0·042	0·558	0·213	0·344	0·557	+0·001
260	0·529	0·041	0·488	0·188	0·282	0·470	+0·018
255	0·498	0·040	0·458	0·235	0·216	0·451	+0·007
250	0·552	0·040	0·512	0·382	0·164	0·546	−0·034

Extinction due to: (1) Actual rhodopsin solution.
(2) Rhodopsin chromophore.

Extrapolation making the assumptions mentioned in text (p. 297).
(3) Difference between (1) and (2).
(4) Calculated absorption due to tyrosine.
(5) Calculated absorption due to tryptophan.
(6) Sum of (4) and (5).
(7) Difference between (3) and (6).

* (4) and (5) were calculated from these figures using the equations given in the text (p. 297).

In Table 4 is set out the results of an analysis of the curve shown in Fig. 2. The absorption due to tyrosine and tryptophan at 280 mμ. has been calculated and, from the curves of pure tyrosine and tryoptophan, the absorption due to these substances has been calculated at other wavelengths. When this absorption, calculated to be due to tyrosine and tryptophan, is subtracted from the protein curve (= rhodopsin less absorption due to the rhodopsin chromophore) the differences are small and are both positive and negative. It may be concluded that, within these limits, the absorption of rhodopsin can be accounted for in terms of the rhodopsin chromophore, tyrosine and tryptophan. If one assumes the data given by Collins & Morton (1950) for the $E^{1\%}_{1\,cm.}$ of rhodopsin (= 6·6 at 500 mμ.), tyrosine (= 78 at 280 mμ. at pH 9·2) and tryptophan (= 262 at 280 mμ. at pH 9·2) the approximate amounts of tyrosine and tryptophan as a percentage of the dry weight of rhodopsin are 6 and 3 respectively. The percentage of tyrosine and tryptophan in other absorption in the region 250–700 mμ. and that the solutions are sufficiently pure for chemical analyses to be meaningful.

SUMMARY

1. Various methods for the separation of rod outer segments from cattle retinas have been investigated, attention being paid to the optical purity of the rhodopsin solutions obtained by extraction of the rod outer segments.

2. A new method has been developed depending on the fact that cattle rods have the same density as 0·88 M-sucrose. The rods are separated by centrifuging them in a sucrose solution in which a density gradient has been established.

3. Various treatments of the isolated rods have been tried in order to eliminate impurities. The best results have been obtained by treating with 4% (w/v) alum solution for 1 hr. followed by 40% (w/v) neutralized formaldehyde solution for 10 min.

4. The effects of these various treatments on the appearance of the rods has been followed microscopically.

5. Very pure cattle rhodopsin solutions have been obtained showing maxima at 498, 345 and 275 mμ. The last band can be accounted for by the presence of tyrosine and tryptophan only. Making some plausible assumptions, rhodopsin appears to contain 6% tyrosine and 3% tryptophan on the dry weight of the protein.

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REFERENCES

Bliss, A. F. (1950). *Fed. Proc.* **9**, 12.
Collins, F. D., Love, R. M. & Morton, R. A. (1952). *Biochem. J.* (in the Press).
Collins, F. D. & Morton, R. A. (1950). *Biochem. J.* **47**, 3.
Krause, A. C. & Sidwell, A. E. (1938). *Amer. J. Physiol.* **121**, 215.
Lythgoe, R. J. (1937). *J. Physiol.* **89**, 331.
Saito, Z. (1938). *Tohoku J. exp. Med.* **32**, 432.
Schneider, E. E., Goodeve, C. F. & Lythgoe, R. J. (1939). *Proc. roy. Soc.* A, **170**, 102.
Sjöstrand, F. S. (1949). *J. cell. comp. Physiol.* **33**, 383.
Wald, G. (1949). *Docum. ophthal.* **3**, 94.
Wald, G. (1951). *Science*, **113**, 287.

Estimation of Protamine and Insulin in Protamine Zinc Insulin

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In a recent publication, Franklin & Quastel (1950) reported that mixtures of proteins could be separated into their individual components by paper chromatography using solutions of sucrose or sodium potassium tartrate for development. The difficulty of locating the position of the protein zones was overcome by adding haemin to the protein mixture and then streaking the paper with a solution of benzidine and hydrogen peroxide. Jones & Michael (1950) separated proteins on columns of cellulose by development with buffer solutions containing ammonium sulphate and located the position of the individual components by treatment with suitable dyestuffs which stained the zones occupied by the proteins, but did not give coloured spots with amino-acids or peptides. More recently, Papastamatis & Wilkinson (1951) used bromothymol blue and tetrabromophenolphthalein ethyl ester to indicate the position occupied by proteins in paper chromatograms. The publication of this last paper prompts us to report our own work on the chromatographic separation and estimation of protamine and insulin.

The initial experiments on the separation of insulin and protamine by paper chromatography were carried out with strips of Whatman no. 1 filter paper 2 cm. wide, using 10 μl. spots of a solution containing 1% protamine sulphate and 1% insulin (w/v). The strips were developed in a downwards direction in the usual way according to the method of Consden, Gordon & Martin (1944) with the phases of a mixture (by vol.) of *n*-butanol (40%), glacial acetic acid (10%) and water (50%), a mixture generally used in the paper chromatography of amino-acids. Solway purple, as recommended by Jones & Michael, was used for indicating the position of the protein bands. Two bands were formed, one due to protamine at the point of application and the other due to insulin, some 6 cm. below the point of application; the solvent front travelled about 25 cm. The separation was not entirely complete, however, and a number of other solvent mixtures were tried. Better results were eventually obtained with the upper phase obtained by equilibrating a mixture of *n*-butanol and glacial acetic acid (3:1 by vol.) with an equal volume of water. This resulted in the formation of two well-defined bands with R_F values of 0 and 0·43 respectively, and this mixture was used in all subsequent work.

The estimation of substances separated by paper chromatography often presents considerable difficulty, although fairly satisfactory methods of estimating individual amino-acids on paper chromatograms have recently been described. It occurred to us that a protein might be estimated with a fair degree of accuracy by the method of retention analysis described by Wieland & Fischer (1948). These workers showed that if a spot of an amino-acid solution was placed near the edge of a