2. In all the preparations studied the anaerobic rate of reduction of cytochrome *c* was greater than that under aerobic conditions. It is considered that oxygen and oxidized cytochrome *c* compete with one another as hydrogen acceptors in the xanthine oxidase system. This is contrary to the findings of Horecker & Heppel (1949).

3. The cytochrome *c* of heart-muscle preparation was found to be reduced by hypoxanthine in the presence of purified milk xanthine oxidase more slowly than the purified, extracted pigment.

4. Phosphate, glyoxaline and glycine buffers, pH 7·3, and sodium and potassium chloride inhibited the rate of reduction of cytochrome *c* by hypoxanthine and xanthine oxidase considerably. Phosphate buffer, pH 7·3, 3×10^{-3} M, inhibited the anaerobic reduction by 44 %.

5. The possibility that reduced xanthine oxidase is not oxidized directly by oxygen in animal tissues is discussed.

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Studies in Rhodopsin

5. CHEMICAL ANALYSIS OF RETINAL MATERIAL

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The chemical composition of the specialized tissues involved in vision is not well established. In respect of low-intensity or scotopic vision, the need is for analysis of the rod outer segments and of rhodopsin, that is of the receptor end-organ and the receptor substance. Retinas themselves have been analysed for various constituents, but the picture is very incomplete and much more work is needed. Rod outer segments (cf. Lythgoe, 1937; Saito, 1938) have not until recently been separable in amounts adequate for analytical work, but appreciable quantities can now be obtained (Collins, Love & Morton, 1952).

Krause (1937) showed that rhodopsin solutions contained phosphorus and choline, suggesting the presence of lecithin. Broda, Goodeve & Lythgoe (1940) reported that, in rhodopsin solutions, 20 % of the weight of solute could be ascribed to phospholipin. Wald & Ishimoto (1946) extracted rod preparations with light petroleum until no further phosphorus-containing material went into solution. Nevertheless, on exposing the extracted rod preparation to light, phospholipin in appreciable quantity was set free, presumably as a result of the decomposition of rhodopsin, and could then be extracted.

Collins & Morton (1950*b*) suggested that nucleotides, probably as nucleic acid, were present in rhodopsin solutions. Ehrlich & Dische (1950) analysed whole retinas from several species of animals for nucleic acid and found a considerable difference from other tissues. They also found that the amounts and proportions of deoxypentose- and pentose-nucleic acids (DNA and PNA) did not vary as between dark and light adapted eyes.

It was decided, therefore, to study the distribution of phospholipin and of nucleic acid in retinas and isolated rod outer segments. For this, the fractionation procedure of Schmidt & Thannhauser (1945) has been followed.

EXPERIMENTAL

Materials. Cattle retinas were obtained from the local abattoir, and the rod outer segments were prepared as described by Collins *et al.* (1952). Some experiments were done using *Rana esculenta* imported from Holland.

Analytical methods. For the analysis of rods, a suspension was prepared and samples used for various determinations. For the estimation of the wet weight, a sample of the rod suspension was centrifuged in a weighed tube and then left to drain. In order to determine the dry weight, the tube containing the rods was dried at 100° until the weight was constant. Another sample was taken and the rhodopsin present was extracted with 1% (w/v) aqueous digitonin after treatment with 4% (w/v) K_2SO_4, $Al_2(SO_4)_3$, $24H_2O$. The amount was determined spectroscopically after mixing with an equal volume of borate buffer (0·05 M, pH 9·3) (Collins & Morton, 1950*a*). The remainder of the rod suspension was used for the determination of the P fractions. For this, the fractionation procedure of Schmidt & Thannhauser (1945) was used; this yielded five fractions; acid-soluble, lipid, pentosenucleic, deoxypentosenucleic and 'phosphoprotein' P. Centrifuging was used instead of filtration. The P was estimated after $HClO_4$ digestion by the method of Berenblum & Chain (1938). The inorganic P, equivalent to the phosphoprotein in the Schmidt & Thannhauser fractionation, was determined by the method of Delory (1938).

RESULTS

Analyses of retinas and rod outer segments

The figures shown in Table 1 give the distribution of phosphorus in retinas from cattle and frogs, and also in the corresponding rod outer segments. These were prepared as described by Collins *et al.* (1952) using the sugar gradient method.

Collins & Morton (1950*b*) conclude that the 'molecular' extinction coefficient of rhodopsin is about 100000 (one 'molecule' = one chromophore) and that one chromophore contains two C_{20} units. Hence one can calculate the amount of vitamin A (mol.wt. 286) equivalent to the rhodopsin present in the solution. As will be seen from Table 1, the rhodopsin content of the rods is twice that of the whole retinas, on a dry weight basis.

The phosphorus fractions show two interesting results: (i) the amount of deoxypentosenucleic acid in rods is extremely low; this agrees with the fact that the rod outer segments have been detached from the rod nuclei; (ii) the amount of phospholipin has been concentrated in rod outer segments almost as much as the rhodopsin, suggesting that the two may be associated.

The other phosphorus fractions do not show such striking differences; the acid-soluble phosphorus and the pentosenucleic acid show decreased concentrations in rods as compared to whole retinas, whilst the 'phosphoprotein' fraction, the significance of which is not clear, is small in both cases and not much altered.

The figures for frog retinas and rod outer segments are not so complete, but are similar. By following the accepted convention that phospholipins contain 4% phosphorus, and that the deoxy-

Table 1. *Composition of retinas and rod outer segments*

(The results are expressed as mg./100 g. fresh tissue and as mg./100 g. dry tissue, shown in brackets.)

Tissue	Dry matter g./100 g. wet tissue	Rhodopsin as vitamin A*	Phosphorus fractions: Acid-soluble	Phospholipin	DNA	PNA	'Phosphoprotein'	Ratio of PNA/DNA
Cattle, whole retinas	14·1	5·5 (39)	61·2 (434)	92·5 (656)	59·7 (423)	20·9 (148)	5·5 (39)	0·35
Cattle, rod outer segments	27·2	22·6† (83)	33·5 (123)	320·0 (1108)	0·8 (3)	17·5 (64)	9·1 (33)	21·9
Ratio of the value for rods/value for whole retina	—	4·1 (2·1)	0·55 (0·28)	3·5 (1·8)	0·013 (0·007)	0·84 (0·43)	1·7 (0·85)	—
Frog, whole retinas	17·0	1·35‡ (7·9)	48·0 (282)	87·8 (517)	63·5 (374)	27·1 (160)	7·2 (42)	0·43
Frog, rod outer segments	—	—	5·54§	41·2§	5·4§	15§ (PNA + 'Phosphoprotein')		2·8

* Assuming the data given by Collins & Morton (1950*b*).
† The rhodopsin solution obtained from these rods had $P_{400\,m\mu.} = 31$ (Collins *et al.* 1952).
‡ Average yield from a number of separate experiments.
§ In μg. These rods were from 120 frog eyes, but owing to loss of material during preparation there was insufficient for weighing.

The amount of vitamin A was calculated as follows. The rhodopsin was extracted, as described above, from a number of whole retinas or a sample of a rod suspension, and the absorption spectrum measured before and after bleaching the solution with light. The difference between the readings at 500 mμ. is due to the rhodopsin chromophore.

pentose- and pentose-nucleic acids and the acid-soluble fractions all contain approximately 10% P, the amounts of these compounds on a dry weight basis can be calculated. The results are shown in Table 2. The most notable feature is the high proportion of phospholipin, nearly 30% in the case of the cattle rod outer segments.

Table 3 shows the distribution of phosphorus in a number of rod preparations; these have been arranged in order of the optical purity of the rhodopsin solutions prepared from the corresponding rod preparation. It is significant that the rod preparations which yield the purest rhodopsin solutions contain the lowest proportion of deoxypentose nucleic acid. As rod outer segments should contain no nuclear material, deoxypentose nucleic acid should also be absent. Its presence, therefore, indicates some contamination with nuclei. For the other constituents, the findings are inconclusive.

Table 2. *Composition of retinas and rod outer segments*

(The results are expressed as g./100 g. of dry tissue.)

	Cattle		Frog,
	Whole retinas	Rods	whole retinas
Rhodopsin (as vitamin A)	0·039	0·083	0·0079
Acid-soluble phosphorus compounds (assumed 10% P)	4·3	1·2	2·8
Phospholipin (assumed 4% P)	16·4	27·7	12·9
Deoxypentose nucleic acid (assumed 10% P)	4·2	0·03	3·7
Pentosenucleic acid (assumed 10% P)	1·5	0·6	1·6

Effect of various subsidiary treatments. Table 4 shows the effect of alum treatment on the phosphorus distribution in rhodopsin solutions. If the alum treatment is omitted, the solutions contain much less phospholipin than solutions prepared from alum-treated rods, and for pentosenucleic acid there is an even larger difference. For rods which have not been treated with alum, most of the pentosenucleic acid remains insoluble in aqueous digitonin, whereas, after treatment with alum, most of the pentosenucleic acid becomes soluble. It must be concluded that treatment with alum splits off phospholipin and pentosenucleic acid from the protein and denatures it. The resulting rhodopsin solution is, however, purer. The accompanying non-rhodopsin protein is presumably denatured and is thus less soluble in digitonin solutions.

In preliminary experiments it had been found that if cattle rod outer segments were ground with quartz, and the resulting suspension subjected to differential centrifugation, the rhodopsin was fairly evenly distributed between the fractions. It was thought worth while to examine the distribution of phosphorus in rods after differential centrifugation.

Rod outer segments obtained from sixty cattle eyes were homogenized with 5 ml. of 0·25 M-sucrose, in a glass Potter-Elvehjem homogenizer. The homogenate was then made up

Table 3. *Distribution of phosphorus in rods contaminated to varying degrees*

(*P* is the ratio of the extinction at 400 mμ. to that at 500 mμ. of the rhodopsin solution prepared from a sample of the total rod suspension. The other figures give the amount of phosphorus in the fraction as a percentage of the total phosphorus.)

P	Acid-soluble	Lipid	DNA	PNA	'Phosphoprotein'
30·6 (best)	8·9	81·0	0·2	5·6	2·6
30·6	8·8	81·0	0·2	4·3	2·2
33·0	1·7	88·0	0·7	6·2	3·1
34·0	22·0	66·0	1·0	7·1	4·2
35·6	25·6	63·8	1·3	8·1	1·2
35·6	25·2	65·0	1·3	7·0	1·1
45·0 (worst)	7·9	79·6	1·6	8·7	2·1

Table 4. *Distribution of phosphorus in digitonin extracts of cattle rod outer segments*

(1% aqueous digitonin was used as extractant, 4% alum solution was used to harden some of the rods. All figures represent the P in a fraction as percentage of the total P.)

	Acid-soluble	Lipid	DNA	PNA	'Phosphoprotein'	Total
Alum-treated, extract	64*		†	10·1	3·5	77·6
Alum-treated, residue	3·7	15·3	†	0·4	3·0	22·4
Not alum-treated, extract	29·8*		†	1·3	4·1	35·2
Not alum-treated, residue	5·4	45·7	†	7·1	6·4	64·6

* It proved impossible to effect a quantitative separation of the acid-soluble and lipid P in the presence of digitonin and hence the combined total is given.

† Very small—less than 0·5% of total P.

to 10 ml., and 2 ml. were removed for the estimation of the P distribution in the whole homogenate. Then 7·5 ml. were taken and centrifuged for 10 min. at 600 *g*. The precipitate was resuspended in 1 ml. of 0·25 M-sucrose and recentrifuged. The precipitate was designated fraction 1. The combined supernatants were then centrifuged for 60 min. at 2000 *g*, and the precipitate was washed with 1 ml. of 0·25 M-sucrose and recentrifuged. This precipitate was fraction 2. Fraction 3 was obtained by centrifuging for 75 min. at 10000 *g* the combined supernatant and washing from fraction 2. Fraction 4 was the supernatant from fraction 3.

The phosphorus distribution of each of these four fractions is shown in Table 5. Fraction 2 contains 61 %, and fraction 3, 28·8 %, of the total phospholipin. Fraction 4, the supernatant, contains 78·9 % of the acid-soluble phosphorus. Apart from this there appears to be no great accumulation of phosphorus compounds in any one fraction.

Two further experiments were carried out to see if it was possible to remove the phospholipin or pentosenucleic acid from rods, and if this would improve the purity of the rhodopsin solution obtained from them.

The rod outer segments from twenty-nine cattle eyes were dried at − 5° in a high vacuum. This took about 70 min. The powder was then ground up in light petroleum (b.p. 40–60°). After an interval, the mixture was centrifuged and the solvent decanted. The dried powder was re-extracted, in this manner, seven times.

The parent rods contained 15·6 μg. phospholipin phosphorus per retina and the combined light petroleum extracts contained 2·11 μg. P per retina. The value for $P_{400\,m\mu.}$ (Collins *et al.* 1952) for the rhodopsin solution, obtained by extracting the solvent-extracted rods was 79, compared with a value of 47 for the rhodopsin from untreated rods.

Nucleoprotein can be dissolved in strong salt solutions; thus Jeener (1948) extracted nucleoprotein from mitochondria using Edsall's solution (0·6 M-KCl, 0·01 M-Na_2CO_3, 0·04 M-$NaHCO_3$). It seemed worth while to try this technique on the rod outer segments especially since it was found that Edsall's solution did not destroy the rhodopsin. A preparation was obtained from cattle rod outer segments after homogenization; the fraction used was not sedimented in 10 min. at 600 *g* but was thrown down in 60 min. at 2000 *g*, using 0·25 M-sucrose as the medium. This material was then extracted with several successive amounts of Edsall's solution for 3 hr. at room temperature and for 8·5 hr. at 0°.

The amount of pentosenucleic acid phosphorus decreased from 19·0 μg. per sample to 9·9 μg. for an equivalent sample after extraction. The rod fragments before and after treatment with Edsall's solution yielded rhodopsin to digitonin solution, but the removal of nucleoprotein did not improve the optical purity.

Table 5. *Distribution of phosphorus in cattle rod outer segments after homogenization*

(The rod outer segments from sixty cattle eyes were homogenized in 0·25 M-sucrose. The figures given are the amounts of P expressed as μg./retina.)

	Acid-soluble	Phospholipin	DNA	PNA	'Phosphoprotein'	Totals
Whole homogenate	3·00	23·10	0·19	1·70	0·84	28·83
Fraction 1 (600 *g* for 10 min.)	0·07	0·86	0·04	0·20	0·08	1·25
Fraction 2 (2000 *g* for 60 min.)	0·40	13·70	0·07	0·59	0·05	14·81
Fraction 3 (10000 *g* for 75 min.)	0·26	6·50	0·04	0·38	0·07	7·25
Fraction 4 (supernatant)	2·73	1·43	0·07	0·55	0·06	4·84
Totals	3·46	22·49	0·22	1·72	0·26	28·15

DISCUSSION AND CONCLUSIONS

Erhlich & Dische (1950) examined the retinas of rabbits and steers, and obtained values for deoxypentosenucleic acid of 396–1240 mg. % and for pentosenucleic acid of 95·4–232 mg. % on a wet weight basis. If it is assumed that the nucleic acids contain 10 % phosphorus, then the present results correspond to about 600 and 250 mg. % respectively for both cattle and frog retinas. This agreement is very satisfactory especially as Erhlich & Dische used a different method of estimation.

As the above authors point out, retinas seem to be very different from most other tissues. The value of the ratio PNA/DNA as given by Davidson (1950) varies from 8·1 for ox pancreas to 0·4 for thymus. Hence retinas with PNA/DNA equal to 0·4 share with thymus the lowest recorded values of this ratio. The amount of PNA phosphorus varies (Davidson, 1950) from 185 mg./100 g. to 20 mg./100 g. Again the retina with 25 mg./100 g. has nearly the lowest recorded amount of pentosenucleic acid. The figure for DNA phosphorus, 60 mg./100 g., is intermediate and represents quite an average concentration. However, these comparisons are complicated by large variations in the percentage dry matter in various tissues. The amounts of acid-soluble phosphorus and phospholipin are not greatly different from those found in liver. The amount of 'phosphoprotein' appears to be much less than in liver—about one-fourth on a dry weight basis.

The high proportion of phospholipin in rod outer segments, about 30 % on the dry weight, is noteworthy. According to Schneider (1946), rat-liver mitochondria contain 18 % on the dry weight and this appears to be the highest recorded figure. The amount of pentosenucleic acid appears to be quite low, less than 1 % on the dry weight, and it is conceivable that this represents contamination by other retinal fragments.

In order to interpret the work on isolated rod outer segments it is informative to consider the work of Sjöstrand (1949) using the electron microscope. He showed that rod outer segments of a guinea pig retina are composed of about 2000 disks, each being about 6–9 mμ. thick and 2 μ. in diameter. When rods break up, the fragments contain variable numbers of disks—from hundreds down to less than ten. Hence it would be expected that if rod outer segments were fragmented, without, however, damaging the individual disks, a continuous range of fractions would be obtained, uniform in composition. However, two other facts may modify this conclusion. The first is that there is reported to be a space between the disks which may contain water-soluble materials; secondly, nodules are apparent on the surface of the disks, which may be detached during homogenization, unless, of course, they are artifacts arising from electron microscopy.

In the present work the results of homogenization and differential centrifugation of cattle rod outer segments indicate that the four fractions obtained are not homogeneous, although the rhodopsin appears to be fairly evenly distributed. The supernatant (fraction 4) has the largest proportion of acid-soluble phosphorus, suggesting that some cytoplasmic fluid is in fact contained between the disks. Fractions 2 and 3 are similar and are notable for the fact that 90 % of the phospholipin of rods is in these two fractions. If fraction 1 contained the larger tissue fragments, such as intact rods, the rod fibres (Sjöstrand, 1949) and other contaminating material, it would seem logical to conclude that fractions 2 and 3 consisted largely of the disks, perhaps as aggregates of various sizes. In that case 90 % of the phosphorus in the disks will be as phospholipin. It was unfortunately not possible to obtain the weight of the fractions and at present it can only be said that the phospholipin, on a dry weight basis, must exceed 30 %.

The results of these chemical analyses appear to be in harmony with Sjöstrand's description of rod outer segments and suggest that the material of the disks is largely lipoprotein.

SUMMARY

1. The distribution of phosphorus compounds, acid-soluble materials, phospholipin, deoxypentose- and pentose-nucleic acids and phosphoprotein, has been recorded for cattle and frog retinas and rod outer segments.

2. Rod outer segments contain little or no deoxypentose nucleic acid and about 30 % phospholipin on the dry weight.

3. Cattle rod outer segments have been fragmented in a homogenizer and four fractions obtained by differential centrifugation. An explanation of the results is given in terms of the structure of rod outer segments proposed by Sjöstrand (1949).

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