reducing power of steroids. The low pH of the reagent, which is responsible for hydrolysis of the cellulose, cannot be raised materially without seriously affecting the sensitivity. The latter depends on the formation of the yellow arsenomolybdate complex which requires a large excess of acid.

In the experience of the author, variations of the blank value and serious disagreement between duplicate determinations are largely due to contamination of the chromatogram strips with extraneous reducing matter. It is therefore apparent that a minimum of handling of the paper strips and scrupulous cleanliness on the bench and in the laboratory atmosphere are essential for satisfactory operation.

In spite of its inherent weaknesses, the arsenomolybdate method is capable of furnishing valuable information on the composition of adrenal extracts. It is hoped eventually to correlate the results obtained by quantitative chromatography with those of biological assays, thus enabling an estimate to be made of the potency, as well as the steroid distribution, of a particular extract.

SUMMARY

1. A method is presented for the quantitative evaluation of adrenal extracts in which the mixture of cortical steroids is resolved into its components by paper chromatography and the chromatogram is incubated with arsenomolybdate reagent. Steroids containing a ketol side chain or having an $\alpha\beta$-unsaturated 3-keto structure quantitatively reduce the reagent to molybdenum blue.

2. The blue zones of the developed chromatogram corresponding to the various cortical steroids are eluted and the blue complex is determined in a photoelectric colorimeter.

3. About 5–100 μg. of reducing steroid can be determined with an accuracy of $\pm$ 10 %.

4. The reducing steroids are determined in two synthetic mixtures and two adrenal extracts.

The author wishes to thank Dr Karl Folkers of Merck and Co. Inc., U.S.A., for the generous gift of some of the steroids used in this investigation; and the Directors of Allen and Hanburys Ltd., for permission to publish this communication.

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Studies in Rhodopsin

6. REGENERATION OF RHODOPSIN

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The history of visual pigments has been reviewed on numerous occasions, e.g. Collins & Morton (1950) and Wald (1951). Under the action of light the rhodopsin content of the eye decreases to an extent depending on time and intensity, whereas in darkness it gradually approaches a maximum. The photochemical destruction ('bleaching') is, however, normally incomplete (Lythgoe, 1940) as rhodopsin is found in light-adapted eyes. The regeneration of rhodopsin, in terms of dark adaptation in the living eye, approaches completion in 30 min. and is almost complete in 45 min. Both vitamin A deficiency (Hecht, Hendley, Frank & Haig, 1946; Medical Research Council, 1949) and anoxia (Hecht & Mandelbaum, 1940) are known to increase the time necessary for dark-adaptation.

Collins (1951) submitted a brief communication to the Biochemical Society reporting work on regeneration of rhodopsin. These experiments used as starting points those of Zewi (1939) in his important quantitative extension of the pioneer work of Kühne (1878). In the present work *in vitro* preparations of eye tissues have been substituted for excised frog eyes, with exposed optic cups, as used by Zewi. Regeneration of rhodopsin might be either an endergonic or exergonic process. If the latter were the case the reaction would be spontaneous, while if the former were true it would have to be coupled to

an exergonic reaction. It was felt that the best results would be obtained by assuming the more complex alternative, and, if regeneration were achieved, the conditions could then be simplified until the minimal requirements were known. Retinas (or retinas plus choroids) from either frogs or rats were ground up and suspended in a 'complete' medium, i.e. one capable of maintaining oxidative phosphorylation. In this way it was hoped to favour the coupling of exergonic systems to the assumed endergonic regeneration. The capacity for regeneration of rhodopsin after bleaching was ascertained, and various components of the medium were then withheld one at a time.

EXPERIMENTAL

Materials

'Complete' medium. Table 1 shows the composition of the medium used. This is very similar to one used by Potter (1945) except that vitamin A has been included.

Vitamin A solution. Crystalline synthetic vitamin A alcohol (Hoffmann La Roche) was added to a solution of Dispersol A (Imperial Chemical Industries Ltd.) or Tween 80 (Honeywell and Stein Ltd., 21 St James's Square, S.W. 1). (*a*) Dispersol A: a few mg. of the vitamin were added to water (5–10 ml.) and one drop of detergent. The mixture was shaken at 50°, until the vitamin was thoroughly dispersed. (*b*) Tween 80: the detergent was dissolved in $CHCl_3$ and the solution added to a few mg. of vitamin A. When the vitamin had dissolved, $CHCl_3$ was removed under reduced pressure and the desired volume of water was added (cf. Bliss, 1951).

To determine the concentration of vitamin A in such aqueous dispersions a small portion was diluted with a known volume of ethanol to bring the intensity of absorption at 320–330 mμ. within the range of the photoelectric spectrophotometer. The absorption curve in the region 310–340 mμ. was plotted and $E_{max.}$ corrected where necessary for irrelevant absorption (Cama, Collins & Morton, 1951).

Adenosinetriphosphate (ATP). This was purchased either as the barium salt (Boots Pure Drug Co., Ltd.) or as the sodium salt (L. Light and Co., Ltd.). The barium salt was converted to the potassium salt after precipitation of $BaSO_4$ and neutralization of the acid with KOH (Bailey, 1949).

Diphosphopyridine nucleotide (DPN). This was supplied by the Schwarz Laboratories (New York).

Cytochrome c. This was obtained from Evans Medical Supplies, Ltd. It was dialysed before use and standardized as described by Potter (1945).

Digitonin solution. The solution (1% w/v in water) was prepared by boiling the requisite amount of digitonin with water and cooling as soon as it had become clear.

Buffer solution (pH 9·3). This consisted of 19 g./l. of $Na_2B_4O_7.10H_2O$.

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

Methods

Animals. Eye tissues were obtained from frogs (*Rana esculenta*) and from rats, both albino and piebald.

Dissection. To dissect the eyes an incision was made close to the junction of the retina and conjunctiva. The incision was then continued with a pair of scissors all around the ora terminalis, thus enabling the cornea, lens, aqueous humour and vitreous humour to be removed. Using a pair of curved forceps the retina, choroid and pigment epithelium were then scraped out and transferred to a centrifuge tube.

Procedure. At least five animals were needed for one experiment, five right eyes serving as a control to the five left eyes. After dissection 0·5 ml. of the medium was added to each lot in a centrifuge tube and the mixture bleached for 1 min. with a photoflood lamp. The retinas, choroids and pigment epithelia were then ground up with a glass rod and finally incubated in the dark for 2 hr. In the case of the frog tissue, moist air was blown into the mixture kept at room temperature. This agitated and aerated the suspension. Moist air and, in a later experiment, moist N_2 were both obtained by bubbling the gas through water, thus preventing the suspension from going dry. With rat tissues, the tubes containing the retina, choroids and medium were stoppered and shaken in a bath kept at 37°. All experiments were done in pairs: e.g. (1) unbleached vs. bleached and then regenerated, (2) bleached and regenerated vs. bleached and not incubated, and (3) bleached and regenerated with vs. without ATP.

Extraction and determination of rhodopsin. Alum solution (4%, w/v) was added to each tube and the mixture left to stand in the dark for 1 hr. It was then centrifuged, the supernatant liquor was discarded and the precipitate washed once with saline solution (0·9%, w/v, NaCl in water). With frog tissues the digitonin solution (usually 0·5 ml.) was then added and left for 1 hr. at room temperature. The solution was again centrifuged and the supernatant liquor poured off and mixed with an equal volume of buffer solution (pH 9·3). This mixture was recentrifuged before being placed in the cell for spectrophotometric examination at 500 mμ. or, occasionally, over the range 400–600 mμ. With rats, usually 1 ml. of digitonin solution was used for the extraction of rhodopsin. No borate buffer was added since it caused precipitation of some of the rhodopsin. The mixture was then centrifuged and the digitonin extract examined as for frogs. The solution was then 'bleached' by exposure to intense white light and the absorption remeasured. The difference in the intensities of absorption at 500 mμ. was taken as a measure of the rhodopsin content. A 1 cm. cell was used throughout. When the volume of rhodopsin solution was less than 2 ml. the cell was modified. Two pieces of thin glass cut to fit inside the cell were placed on either side of the light path. In this way the volume of liquid necessary was reduced without interfering with the light falling on the photo-cell. The compensating cell, in all cases, contained distilled water, since only the difference spectrum was important. The yield of rhodopsin as recorded in this paper means:

$$\frac{(\text{Extinction at } 500\,\text{m}\mu\text{. due to rhodopsin}) \times (\text{volume of soln})}{(\text{Number of retinas})}.$$

RESULTS

Experiments with frogs

Using the complete medium (Table 1) regeneration with frog eye tissue could be demonstrated. Experiments were then carried out to determine the necessity or otherwise of components of the

medium for optimal regeneration and the results are shown in Table 1. The figures given are averages of a number of experiments. Both vitamin A and ATP were necessary; in the absence of either the yield fell to about 50 %.

Table 1. *The results obtained from a series of experiments on the regeneration of rhodopsin using frog retinas and choroids*

(Reaction mixtures of 1 ml. were prepared and used as described in the experimental section. Final concentrations of reactants were: potassium succinate, 0·02 M; phosphate buffer, pH 7·4, 0·034 M; magnesium sulphate, 0·005 M; nicotinamide, 0·03 M; cytochrome *c*, 1×10^{-5} M; vitamin A alcohol, $2\text{–}8 \times 10^{-4}$ M; diphosphopyridine nucleotide, 5×10^{-5} M; adenosinetriphosphate (ATP), $1{\cdot}3 \times 10^{-3}$ M).

	Yield per eye (fiducial limits for $P = 0{\cdot}05$) (for units see text)	No. of samples	Percentage yield
Unbleached	0·036 ± 0·0037	8	100
Bleached	0·008 ± 0·0022	8	22
Regenerated with complete medium	0·033 ± 0·0033	8	92
ATP not added	0·016 ± 0·0053	3	44
Vitamin A not added	0·018 ± 0·0076	5	50

Table 2. *Regeneration of frog rhodopsin under varying conditions; individual experiments*

(The reaction mixture used was the same as in Table 1 except where indicated.)

	Yield per eye (for units see text)	Percentage yield
—	0·029	100
Nicotinamide and diphosphopyridine nucleotide withheld	0·016	55
—	0·028	100
Cytochrome *c* withheld	0·016	57
—	0·031	100
N_2 used instead of air	0·022	71
Regenerated for 1 hr.	0·026	79
Regenerated for 2 hr.	0·033	100
Time between killing and starting the experiment:		
(*a*) 40 min.	0·027	100
(*b*) 120 min.	0·019	70

A number of further experiments were carried out (Table 2). (i) When either cytochrome *c* or DPN plus nicotinamide was omitted the yield fell to about 55 % of the control in which the complete medium was used. (ii) If moist nitrogen was blown into the mixture instead of air the regeneration fell to 71 %. (iii) The supply of frogs was not large enough to enable a complete regeneration-time curve to be constructed. In one experiment the regeneration after 1 hr. was 97 % of that after 2 hr. (iv) The final experiments illustrate the fact that the amount of regeneration decreases if the eye tissues are kept too long before incubation even if the complete medium is used. Potassium succinate was not needed in the medium.

Experiments with rats

At first, albino rats were used, but it was soon apparent that the amount of rhodopsin in unbleached retinas was low. Regeneration in these experiments was so small as to be negligible. The average yield of rhodopsin for thirty-five albino rat eyes was 0·009, compared to 0·024 for piebald rats. After weaning, eight animals of both types were fed on the standard diet used in the small-animal colony in this laboratory. The amount of vitamin A in the livers was between 600–700 i.u./g. in both cases. The yield of rhodopsin from unbleached eyes of albino rats was 0·008; the corresponding figure for the piebald rats was 0·027. After an initial bleaching followed by incubation at 37° for 2 hr. in the dark, using the 'complete' medium, the yield for the albinos was 0·0025 while the piebald rats gave a yield of 0·017. After this experience only piebald rats were used.

A number of experiments were carried out in which the need for each component of the 'complete' medium was tested. It was found that maximal regeneration could be obtained when the medium contained only phosphate buffer, magnesium ions and vitamin A. The need for magnesium ions was not investigated.

The yield of rhodopsin in twenty-five regeneration experiments was 0·0179 ± 0·0008. The experimental conditions are quite critical. A large number of other experiments failed to give regeneration in that the results did not differ significantly from the yield at zero time, and a combined figure of 0·0075 ± 0·0006 (thirty experiments) was obtained. This figure is not easy to interpret, although various tentative explanations could be offered. From the present point of view it offers a convenient base line for assessing the extent of regeneration. The average yield for the fully dark-adapted eye, using the figures obtained by Collins & Morton (1950) in conjunction with those obtained in ten experiments in the present work, was 0·0236 ± 0·006. The average amount of regenerated rhodopsin is thus 76 % of the rhodopsin content of the dark-adapted living rat eye.

Terner, Eggleston & Krebs (1950) showed that oxidative phosphorylation was possible with cattle retinas in the presence of D-glucose and L-glutamic acid. Adapting their experimental procedure, the left eyes of six rats were dissected on ice and incubated for 2 hr. with a medium containing only phosphate buffer, glucose and glutamic acid. The six right eyes were dissected at room temperature and incubated with the same medium for the same length of time as a control. The results of this

Table 3. *Experiments on the regeneration of rhodopsin showing the effect of dissection at room temperature and at 0°, and the effect of vitamin A and of 2:4-dinitrophenol*

(Basic medium: 0·04 M-glucose, 0·02 M-glutamic acid, 0·034 M-phosphate buffer (pH 7·4). When used, the concentration of vitamin A alcohol was $2–8 \times 10^{-4}$ M and the 2:4-dinitrophenol was 1×10^{-4} M.)

Conditions of dissection	Change in basic medium	Experimental pair* 1	2	3	4
Room temperature	None	0·008	0·012	—	—
0°	None	0·014	0·017	—	—
0°	+ Vitamin A	—	—	0·019	0·020
0°	+ Vitamin A and 2:4-dinitrophenol	—	—	0·014	0·017

* The experimental pairs are entered in the same column.

experiment are shown in Table 3, from which it is apparent that the best regeneration was obtained when the operations, prior to bleaching and incubation, were carried out at a temperature near 0°.

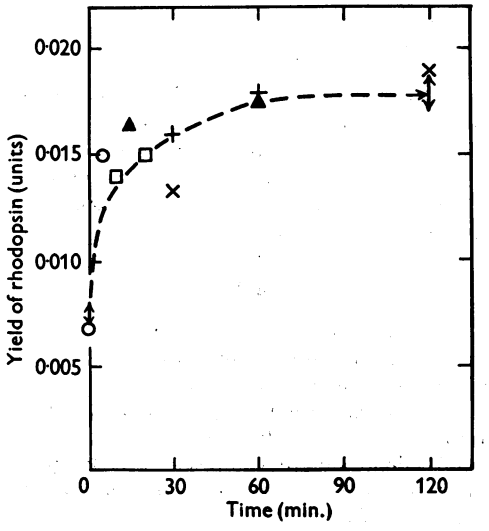

Fig. 1. Relationship between the amount of regeneration using rat retinas and choroids dissected out at room temperature, and the time of incubation in the dark using a medium containing $2–8 \times 10^{-4}$ M-vitamin A alcohol and 0·034 M-phosphate buffer (pH 7·4). The average ranges obtained in a number of experiments, for the amount of rhodopsin at zero time and after 2 hr. incubation, are shown by vertical lines. The remaining points on the curve were determined in pairs (six right and six left eyes in each experiment) and each pair has the same symbol. For definition of rhodopsin units see text.

The addition of 2:4-dinitrophenol appeared to neutralize the beneficial effects of vitamin A. This effect of vitamin A is in agreement with the observation already made that vitamin A is necessary for a maximum regeneration when the dissection procedure is carried out at room temperature.

Attempts were then made to construct a curve relating the amount of regeneration to time of incubation. As it was impossible to do this in a single experiment it was necessary to construct a composite curve using data from five experiments. Since one could not be certain that the rats in the different experiments were comparable, this may account for the rather large scatter of the points obtained. However, it is quite clear that most of the regeneration is complete in 1 hr. (see Fig. 1).

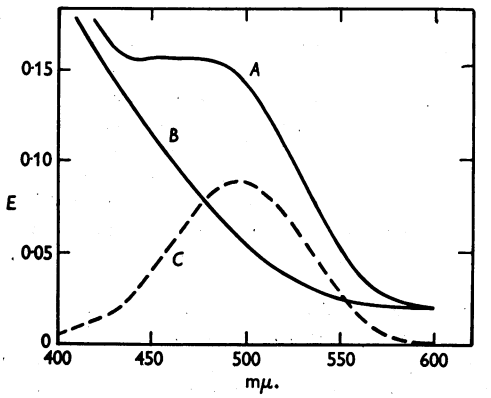

Fig. 2. The absorption spectrum of regenerated rat rhodopsin. Curve *A* is the spectrum of the unbleached solution while curve *B* is the spectrum of the same solution after bleaching. Curve *C* is the difference between *A* and *B* and represents the absorption due to rhodopsin.

Fig. 2 shows the absorption spectrum of a solution of regenerated rhodopsin.

DISCUSSION

Zewi (1939), working with frogs (*Rana esculenta*), completed an extensive analysis of the regeneration of rhodopsin in living animals, and on excised opened eyes. He found that the course of the regeneration in live frogs was influenced by temperature; within the range studied 22·1° was the optimal. In excised open frog eyes no influence of temperature on regeneration was observed. At

7·2° the regeneration proceeded as rapidly in the excised open eyes as in the intact animals at the same temperature. Thus, part of the regeneration in the living frog eye must be susceptible to temperature and this suggests that the regeneration of rhodopsin may follow one of two paths. Further evidence that this is so was obtained by Zewi (1941), who demonstrated that the component of regeneration which is susceptible to temperature is also influenced by drugs. Atropine retarded the process at 22·4°, but had no effect at 8°. This suggests that the energy used for the phase of regeneration sensitive to both temperature and drugs is dependent on living processes. Zewi also demonstrated that the presence of oxygen was necessary for regeneration. Regeneration *in vitro* has been obtained with both species. With frogs, the need for oxygen, vitamin A and a medium capable of supporting oxidative phosphorylation was demonstrated, but rats needed only vitamin A. The retina of the frog differs from the mammalian retina in its dependence on the choroidal rather than the retinal circulation for its nourishment. This difference may be responsible for the difference observed in the medium which will give maximum regeneration in the frog and rat eyes. With rats the necessity for vitamin A has been demonstrated either when the eyes are dissected at room temperature or under ice-cold conditions. The presence of ATP in the medium is not necessary using rat tissue under these latter conditions, but when the rat eyes are dissected at room temperature conflicting results have been obtained; the majority of experiments, however, have been successful in its absence.

The results obtained using 2:4-dinitrophenol make it tempting to draw the conclusion that phosphorylation is necessary for the utilization of vitamin A alcohol. According to the present state of knowledge (Copenhauer & Lardy, 1952) 2:4-dinitrophenol is a more effective inhibitor of phosphorylation coupled with electron transport (reduced diphosphopyridine or triphosphopyridine nucleotide-flavoproteins-cytochromes) than of simple substrate phosphorylation (as in anaerobic glycolysis). In the frog experiments the necessity for ATP in the medium used for a maximum regeneration can be regarded as substantiated. With rats the fact that it is not necessary to add ATP to the medium does not mean that it plays no part either directly or indirectly in the process of regeneration. If an endergonic reaction is concerned in the regeneration of rhodopsin, it is conceivable that the energy required (presumably as ATP) may be obtained from glycolysis.

Wald and his co-workers (Wald & Brown, 1950; Wald & Hubbard, 1950, 1951) have presented evidence concerning the mechanism of rhodopsin synthesis using frogs' eyes (*Rana pipiens*) and cattle eyes. It appears that in all their experiments involving the use of tissue homogenates in the presence of vitamin A the period of incubation used varied from 9 to 12 hr. The present work with frogs and rats has shown that the greater part of the regeneration is over after 1 hr. This agrees closely with the time required for dark-adaptation in the living eye.

The following comments can be made. It is clear from the present work that regeneration of rhodopsin can occur *in vitro* and can be studied in detail. The natural process, which requires vitamin A and is complete in less than an hour, is closely simulated *in vitro*. When eye tissues from rats or frogs are used the experimental work is difficult and tedious, largely because the absolute amounts of rhodopsin are quite small. It is, therefore, a considerable advantage to be able to use cattle retinas not only because they are plentiful but also because they are so much larger.

Wald and his co-workers have published observations on the regeneration of rhodopsin which appear to differ from some of those presented here. Their tentative interpretation also differs, but it is too early to debate this and both experimental approaches need to be continued.

SUMMARY

1. The regeneration of rhodopsin has been studied using isolated retinas with choroids, from frogs (*Rana esculenta*) and rats.

2. With frogs' retinas 92 % regeneration could be obtained using a medium containing vitamin A alcohol, adenosinetriphosphate, diphosphopyridine nucleotide, cytochrome *c*, and nicotinamide. Oxygen was also necessary.

3. Although albino and piebald rats did not differ in respect of the amount of vitamin A stored in the liver, the rhodopsin content of the eyes of piebald rats was more than three times greater than that of albino rats. Moreover, the albinos had extremely low capacity for regenerating rhodopsin.

4. When rat retinas and choroids were dissected out with the minimum delay after death, maximum regeneration (76 %) was obtained using a medium containing only phosphate buffer and vitamin A.

5. The time required for regeneration *in vitro* in rat retinas and choroids is between 30 and 60 min., in good agreement with human dark-adaptation time.

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Spectrophotometric Determination of Fructose-1:6-Diphosphate, Hexosemonophosphates, Adenosinetriphosphate and Adenosinediphosphate

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Spectrophotometric methods are now widely used for following the course of certain enzymic reactions. When the reverse reaction is slight, it is possible to adapt the procedure so that it becomes a specific and usually very sensitive method of measuring the concentration of the reactants. Reactions involving oxidized or reduced diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) have been particularly useful, since the reduced coenzyme absorbs strongly in the near ultraviolet (Warburg & Christian, 1936) and a number of enzymes catalysing the reaction between various intermediary metabolites and the coenzyme can be prepared. Examples are the estimation of pyruvate and malate (Ochoa, Mehler & Kornberg, 1948), *iso*-citrate (Ochoa, 1948), α-ketoglutarate (Kornberg & Pricer, 1951*a*) and glucose-6-phosphate (Ochoa, Salles & Ortiz, 1950; Slein, 1950; Kornberg & Pricer, 1951*b*).

In connexion with investigations of oxidative phosphorylation, a very sensitive method of determining fructose-1:6-diphosphate (HDP) was required. Dr Racker suggested to me that his method (Racker, 1947) of measuring phosphohexokinase activity might be adapted for this purpose. The present paper describes the successful adaptation of this method for the estimation not only of HDP, but also of the hexosemonophosphates (glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate) and adenosinetriphosphate (ATP) and adenosinediphosphate (ADP). It can also be adapted for the measurement of creatinephosphate. A preliminary account of this work has already been published (Slater, 1951).

PRINCIPLE OF METHODS

Procedure A. In the presence of rabbit-muscle fraction *A* (see Methods), HDP reacts with an excess of reduced DPN, according to the following scheme:

(1) HDP → glyceraldehydephosphate + dihydroxyacetonephosphate (*aldolase*),

(2) Glyceraldehydephosphate → dihydroxyacetonephosphate (*triosephosphate isomerase*),

(3) 2 Dihydroxyacetonephosphate + 2 (reduced DPN) → 2 glycerolphosphate + 2DPN (*glycerolphosphate dehydrogenase*).

Overall reaction (*A*): HDP + 2 (reduced DPN) → 2 glycerolphosphate + 2 DPN.