Table 2. *Excretion of arsenic after the injection of arsanil-azo-gelatin and arsanil-azo-globulin*

Exp. no.	Rabbit no.	Injected arsanil-azo-protein: Wt. mg.	Protein	Content of As mg.	Content of arsenic in urine: 24 hr. mg.	48 hr. mg.
1	208	60	Globulin	2·5	0·91	—
	209*a*	50	Gelatin	1·8	1·54	—
2	210	60	Globulin	2·5	0·88	—
	209*b*	50	Gelatin	1·8	1·71	—
3	213	48	Globulin	2·0	0·28	0·06
	214	45	Gelatin	1·6	0·99	0·05
4	215	48	Globulin	2·0	0·47	0·02
	217	56	Gelatin	2·0	0·75	0·07

yielded only negligible quantities of dialysable As compounds.

The rapid elimination of As when administered intravenously as arsanil-azo-gelatin appears to be related to the peculiarities of the gelatin molecule, e.g. its strong tendency to remain in solution and not to be adsorbed. It is assumed therefore, that the failure of arsanil-azo-gelatin to produce antibodies is chiefly due to the fact that its molecules are not deposited at the sites of antibody-formation, but are rapidly eliminated from the organism. Probably this is also the reason for the lack of antigenicity of pure gelatin.

SUMMARY

1. While an intravenous injection of arsanil-azo-globulin into rabbits gives rise to a considerable deposition of arsenic in the liver, an analogous injection of arsanil-azo-gelatin is followed by rapid urinary excretion of arsenic, and only small quantities of arsenic are deposited in the liver.

2. The failure of arsenil-azo-gelatin to act as full antigen is attributed to the insufficient deposition of this substance in the reticulo-endothelial cells, i.e. in the cells producing the antibodies.

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Riboflavin and Riboflavin Adenine Dinucleotide in Ox Ocular Tissues

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Sherman & Sandel [1931] found that vitamin 'G'-deficient rats showed eye changes which they described as 'a very slight keratitis with corneal corpuscles, a few polymorphs and some new formed blood channels in the cornea'. Day, Langston & O'Brien [1931] also observed that rats on a 'G'-deficient diet developed eye lesions. Their animals not only developed a condition which they described as anterior interstitial keratitis, but also cataract. The lens changes in vitamin 'G' deficiency were further investigated by many different workers, but the original observations could not be repeated. The changes in the cornea were not examined in detail until Bessey & Wolbach [1939] and El Sadr [1939] published descriptions of the vascularization of rat corneas in riboflavin deficiency. In the meantime vitamin 'G' had become identified with vitamin B_2 or riboflavin. Sydenstricker, Sebrell, Cleckley & Kruse [1940] described vascularization of the cornea in man in riboflavin deficiency. Patek, Post & Victor [1941] have found that it occurs in pigs, and Street, Cowgill & Zimmerman [1941] have described its occurrence in dogs.

Apart from estimations of the total flavin content of the retinae of different species by v. Euler & Adler [1933; 1934*a*] no determinations of the amount or type of flavin in the different parts of the eye have been published. We have now estimated total flavin and riboflavin adenine dinucleotide in ox eyes in order to see whether a determination of the distribution and type of flavin present would help to explain the sensitivity of the cornea to riboflavin deficiency.

METHODS

(1) *Collection of material*

Ox eyes were removed and put into a refrigerator within 5 min. of the death of the animal. As soon

as the requisite number of eyes had been collected they were taken on ice to the laboratory, dissected, and extracts made of the different parts. The eyes were gently wiped with a damp pledget of cotton-wool. The corneal epithelium was then shaved off with a sharp knife. We found that the best method of removing the retina was to brush it out with a soft paint brush, the choroid could then be taken out with forceps. The aqueous humour, when needed, was removed at the slaughter house with a hypodermic syringe, and immediately cooled on ice. The lens was extracted without its capsule. Ocular conjunctiva was cut off as a very thin strip without adherent fatty tissue, in order to make conjunctiva as comparable with corneal epithelium as possible.

Preparation of extracts. Snell & Strong [1939] recommend extraction of tissues low in riboflavin either with boiling $N/10$ HCl or with a mixture of 3 parts acetone, 1 part N H_2SO_4 under reflux. We found, however, that these methods gave lower values than extraction with H_2O at 80° for 15 min., which is the method used by Ochoa & Rossiter [1939] to prepare extracts for riboflavin adenine dinucleotide estimation. The final method of preparation of tissue extracts for both the microbiological estimation of total riboflavin and the estimation of riboflavin adenine dinucleotide has therefore been as follows. The tissue was weighed, ground with a little sand and extracted with distilled water in a water-bath at 80° for 15 min. The final concentration of tissue was 10 or 5%. The tube was then cooled to 4° and the extract centrifuged. The supernatant, which is frequently cloudy, was used for the estimations.

(2) *Microbiological estimation of total riboflavin*

Total riboflavin was estimated by the microbiological method of Snell & Strong [1939]. A culture of *Lactobacillus helveticus* (kindly supplied by Dr J. G. Davis of the National Institute for Research in Dairying) was used. A few experiments were done with additions to the medium as described by Barton-Wright [1942], i.e. xylose, asparagine, nicotinic acid and pantothenic acid. Although these additions did accelerate the growth of *L. helveticus* to a small extent, they did not affect the values for riboflavin obtained from different extracts of the same tissues. For the rest of the work the simplest medium was used. The growth of the *L. helveticus* in 24–48 hr. was measured by titrating the acid formed with $0{\cdot}1N$ NaOH, using phenol-red as an indicator. A standard riboflavin curve was obtained with each experiment between the limits of 0–0·1 μg. riboflavin and the extracts were diluted, if necessary, to fall within these levels. All extracts, with the exception of tears and meibomian secretion, were estimated in duplicate at three different levels.

(3) *Estimation of riboflavin adenine dinucleotide*

This was estimated by the *d*-amino oxidase technique of Warburg & Christian [1938], using the specific enzyme-protein from sheep's kidney. The pure substance was not available as a standard, so an extract of rat brain was used. Hot aqueous extracts of rat brain consistently contain a mean of $10 \pm 0{\cdot}8$ μg./g. fresh tissue, based on groups of six animals [Ochoa & Rossiter, 1939], and we have assumed a value of 10 μg./g. in constructing a reference curve for the O_2 uptake of 5 mg. of the kidney protein in the presence of *dl*-alanine and varying amounts of rat-brain extract. In assays on extracts of eye tissues the amounts added were always such as to give O_2 uptakes falling on that part of the reference curve for which the increase in uptake was directly proportional to the amount of catalyst added. It is realized that the values we have obtained are approximate only, as the brain extract cannot be as satisfactory a standard as the pure riboflavin adenine dinucleotide.

RESULTS

Table 1 summarizes the results we have obtained by the two methods.

We do not wish to stress the apparent agreement between the riboflavin values obtained by the two separate methods of estimation, as estimations of the flavin contents of other tissues made in the same way did not agree so satisfactorily. The riboflavin values obtained by the *d*-amino oxidase technique, given in Table 1, demonstrate the presence throughout the eye of riboflavin adenine dinucleotide.

Adler & v. Euler [1938] found that free riboflavin was present in an extract, prepared at 0°, of cod's eyes, but the question of the presence of free riboflavin in mammalian retinae is still open. Many fish retinae contain much more total flavin than any known mammalian retina. v. Euler & Adler [1934*a*] found 0·2–0·4 μg. riboflavin/g. wet wt. of human retina. We were able to obtain one normal human retina which was iced within 1 min. of extraction of the eye. The total riboflavin content, estimated microbiologically, was 3·2–5·0 μg./g. wet wt., and estimated by the *d*-amino oxidase technique was 5·0 μg./g. wet wt. Even this higher value is far below the flavin content of some fish retinae, which Adler & v. Euler [1938] found to contain 500 μg./g. wet wt.

v. Euler & Adler [1933] based their contention that the riboflavin in the retina is free on the fact that it is largely dialysable. But Pulver [1940] has shown that the flavin from all tissues becomes progressively dialysable as the time of dialysis increases, and that in order to get a true value for free riboflavin an ultrafiltrate of a press juice of the

Table 1. *Riboflavin in ocular tissues estimated by microbiological and* d*-amino oxidase techniques*

Tissue	Extractive	Riboflavin μg./g. wet wt. unless stated otherwise: Microbiological	*d*-amino oxidase (H_2O extracts only)
Corneal epithelium	*N*/10 HCl	1·76	1·8, 1·9, 2·5, 2·4, 2·3
	N/10 HCl	2·4	2·7
	Acetone, H_2SO_4	1·6	
	H_2O	1·5	1·7
Substantia propria	*N*/10 HCl	0·27	
	Acetone, H_2SO_4	0·18	0·42, 0·26, 0·25
	H_2O	0·24	0·54
Conjunctiva	*N*/10 HCl	0·9	2·6
	H_2O	1·6	
	H_2O	1·4	2·1
	H_2O	2·1	2·1
	H_2O	2·6	2·1
Aqueous humour	*N*/10 HCl	1·0 μg./100 ml.	1·3 μg./100 ml.
	N/10 HCl	0·9 μg./100 ml.	
	N/10 HCl	1·1 μg./100 ml.	
Iris	H_2O	1·4	1·2, 2·2
	H_2O	1·8	2·8
Ciliary body	H_2O	2·0	1·8
	H_2O	1·8	2·6
			1·6, 2·9
Lens	H_2O	0·05	0·28
	H_2O	0·12	0·2
Vitreous humour	Acetone	0·8 μg./100 ml.	
Retina	*N*/10 HCl	0·9	2·8, 2·1, 4·4
	N/10 HCl	1·1	3·9, 3·9
	N/10 HCl	2·1	
	H_2O	4·6	3·6, 3·8
	H_2O	4·3	1·5
	H_2O	4·9	
	H_2O	3·5	4·5
Choroid	*N*/10 HCl	0·9	1·8, 2·5, 2·8
	N/10 HCl	0·9	
	N/10 HCl	1·1	
	H_2O	1·8	2·8
	H_2O	2·9	1·5
		3·2	1·4

In those cases where a dotted line is drawn between the figures, the microbiological and the *d*-amino oxidase estimations were carried out on the same extract.

tissue should be made. Using this method he found, for example, that only 2% of the total flavin of cattle liver was dialysable.

v. Euler & Adler [1934*a*] considered that the retinal flavin was almost entirely confined to the pigment epithelium of the retina. In our experiments histological preparations showed that the pigment epithelium remained attached to the choroid, and none of it was present in the retinal preparations. Yet the flavin content of the ox retina is higher than that of the choroid. Acetone extracts of calf retina made by v. Euler & Adler [1934*b*] gave non-fluorescent extracts, which after standing for 2 days fluoresced blue. This suggests a breakdown of a combined flavin present in the original extract.

We have done a single experiment to see whether bleaching of the retina caused any diminution of its riboflavin adenine dinucleotide content. An excised ox eye was placed in a dish of ice and exposed to direct sunlight for 1 hr. At the end of this time the dinucleotide content of the retina, determined in the usual way, was normal in amount.

In Table 1, the low value for the flavin of the aqueous humour is very noticeable; that for the *substantia propria* of the cornea is also low, while the ocular conjunctiva and the corneal epithelium contain about 2 μg./g. of total flavin. Histological preparations showed that very little *substantia propria* was detached with the corneal epithelium. This accumulation of flavin in the corneal epithelium, which is without a blood supply, led us to investigate the flavin content of the glands of the eye and their secretions.

Table 2. *Riboflavin in ocular glands and secretions, estimated by microbiological and* d*-amino oxidase techniques. H_2O used as extractive throughout*

Tissue	Riboflavin, μg./g. wet wt. unless stated otherwise: Microbiological	*d*-amino oxidase
Meibomian glands	3·9	4·1
		4·3
Lacrymal glands	6·5	6·3
		6·2, 7·2
Meibomian secretion	4·1, 3·3	
	0·0, 3·1	
Tears: Human	0·01 μg.	0·027 μg. Total riboflavin. Vol. of tears, approx. 0·2 ml. in each case
Rabbit	0·03 μg.	
Rat	0·04 μg.	

There are two main lacrymal glands in the ox; both had similar flavin contents, averaging 6·5 μg./g. The meibomian glands of the lids contained about 4 μg./g. These values, in particular that for the lacrymal glands, are higher than those for the tissues adjacent to the corneal epithelium, or indeed for any part of the eye except possibly the retina. We therefore estimated the total flavin content of the eye secretions. Ox eyelids were cut off immediately after death and the secretion from the meibomian glands was expressed by hand. A few mg. of a hard bright yellow waxy substance was obtained from several lids. Too little was obtained to make it feasible to do parallel estimations of total flavin and riboflavin adenine dinucleotide, so that values for total flavin only are given (Table 2). These show that the secretion contains about the same amount of flavin/g. as the gland itself. Miscellaneous estimations of the presence of flavin in the tears of different species have been carried out. The volume of tears obtained was so small that it was only possible to decide whether flavin was present or not. The results show that flavin was present in tears from a normal woman, from rabbits, and from a rat.

It is therefore possible that the flavin of the corneal epithelium is obtained from the lacrymal

secretions rather than from the blood in the limbal loops. The weight of epithelium of an ox eye is approximately 0·15 g., which means that the amount of riboflavin present is 0·3 μg. It seems reasonable to suppose that this amount could be kept replenished by the eye secretions, but we have no evidence that *in vivo* such an absorption of either riboflavin or riboflavin adenine dinucleotide into the cornea does occur. The penetration of the corneal epithelium by either of these substances has not yet been investigated. Histological studies of the eye glands of riboflavin-deficient animals have not been reported, although both deficient and excessive secretion of tears have been noted, so that one cannot tell whether the primary lesion leading to a disturbance of the corneal epithelium with subsequent vascularization of the cornea is in the eye glands and their secretions or not. Deficiency of tears in man may rarely be associated with corneal disturbance, as in *keratoconjunctivitis sicca* described by Bruce [1941], but in general either the meibomian glands or the lacrymal gland may be removed without subsequent damage to the cornea. Under such circumstances the mucous-secreting glands of the conjunctiva still remain.

Johnson & Eckhardt [1940] reported that exposure of rats to sunlight hastened the vascularization of the cornea in riboflavin deficiency. We have investigated the stability to light of riboflavin adenine dinucleotide in ox corneal epithelium. Ox eyes were cooled to 4° and placed in a dish of ice in bright sunlight out of doors for 1 hr. The surface of the cornea was kept moist by occasional drops of ice-cold saline. The riboflavin adenine dinucleotide of the epithelium, estimated in the usual way, showed no diminution during this time. Ox eyes were also similarly exposed for a maximum time of 1 hr. to a G.E.C. Osira ultra-violet lamp placed 8 in. away from the corneal surface. Again no change occurred in the dinucleotide content of the epithelium. Excised epithelium exposed to the G.E.C. Osira lamp also had a normal dinucleotide content after exposure. These results with excised eyes give the impression that the dinucleotide itself in the ox corneal epithelium is relatively stable, but they must not be regarded as contradicting Johnson & Eckhardt's results, where continuous exposure to sunlight *in vivo* resulted in an apparent diminution of the corneal flavin.

DISCUSSION

These estimations of riboflavin and riboflavin adenine dinucleotide in the secretions and different parts of the eye show that the distribution is general, but uneven. The vitreous humour, the aqueous humour and the lens contain very small amounts of flavin. This is in marked contrast to the distribution of ascorbic acid, whose concentration in ox aqueous humour is 20 mg./100 ml. [Müller, 1933], a concentration greater than that in the blood. Birch & Dann [1933] found that ox lens, and Monajukowa & Fradkin [1935] found that the vitreous humour also had a high content of ascorbic acid. The estimations also show that some at least of the retinal and choroidal flavin in the ox is present as dinucleotide.

In riboflavin deficiency in man and animals the cornea is invaded by capillaries at an early stage. Our estimations of the flavin content of the epithelium and the surrounding tissues show that the corneal epithelium has a greater concentration of flavin than the *substantia propria* or aqueous humour, and about the same concentration as the conjunctiva. The source of this flavin in the corneal epithelium has been considered to be the blood in the vessels at the limbus. Our estimations of the flavian content of the eye glands and their secretions show that if the epithelium is permeable to flavin it would be possible for it to obtain it from tears and from the meibomian secretion, and we think that this possibility should be borne in mind in considering the ocular signs of riboflavin deficiency.

SUMMARY

Total riboflavin and riboflavin adenine dinucleotide have been estimated in ox ocular tissues. The lacrymal and meibomian glands contain a greater amount of flavin than does any other part of the eye. The corneal epithelium contains much more than the *substantia propria* or the aqueous humour, and it is tentatively suggested that the corneal epithelium may get flavin from the eye secretions as well as from the blood in the limbal capillaries.

We wish to thank Miss Mann for her interest and help, Dr Carleton for preparing the histological specimens, Dr Rossiter for suggesting that rat brain extract may be used as a standard in estimating riboflavin adenine dinucleotide, and finally the staff of the Oxford and District Co-operative Society, Ltd., whose friendly help made the work possible.

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The Oxalate Content of Blood

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The quantity of oxalate present in blood is still in dispute. Japanese workers give the normal value as 2–4 mg./100 ml. (method of Izumi [1933], Suzuki [1934], Kamiya [1937] and Sato [1941]). On the other hand, Leulier & Dorche [1939] found 0·2–0·5 mg./100 ml. in good agreement with Flaschenträger & Müller [1938]. Voinar & Babkin [1940] failed to isolate oxalic acid from the blood or tissues (as CaC_2O_4), and expressed doubts as to the reliability of the methods then available. Barber & Gallimore [1940] applied the ester method of Dodds & Gallimore [1932] and reported 0·4–0·6 mg./100 ml. of human blood.

The method of Izumi [1933], which depends on the precipitation of cerous oxalate ($Ce_2(C_2O_4)_3$) at *p*H 2·3 from a trichloroacetic acid filtrate, received some support from the previous work of Merz & Maugeri [1931]. These authors found 3–4 mg./100 ml. by direct precipitation from blood filtrate by means of lime water, but Thomsen [1935] and Voinar & Babkin [1940] have claimed that the precipitate obtained does not consist of oxalate. Thomsen [1935] also criticized the Ce procedure used by Suzuki [1934], but this criticism was answered [Suzuki, 1936] and the method has been in use in a modified form ever since. The purpose of the present paper has been to investigate the Ce procedure used by Kamiya, Noye & Sato [1937] and the Ca procedure of Merz & Maugeri [1931], since these two methods agree in yielding high and unconfirmed results. The ester procedure of Barber & Gallimore [1940] has also been examined.

METHODS

(1) *Investigation of the cerous oxalate method* [Suzuki, 1934; Kamiya *et al.* 1937]. Thomsen [1935], following the procedure of Suzuki [1934], could obtain no precipitate from blood filtrates, and pointed out that $Ce_2(C_2O_4)_3$ is soluble in excess of $CeCl_3$. Suzuki [1936] suggested that Thomsen had overlooked or lost the small precipitate of $Ce_2(C_2O_4)_3$ and insisted that special centrifuge tubes were required. Subsequently, Kamiya *et al.* [1937] suggested $Ce_2(SO_4)_3$ as precipitant since an excess of this substance has less tendency to redissolve $Ce_2(C_2O_4)_3$.

In the present experiments the special centrifuge tubes recommended by Suzuki [1936] were employed. With 2 drops of 0·1% $Ce_2(SO_4)_3$ and 3 ml. of a solution of $Na_2C_2O_4$ equivalent to 1·33 mg. $H_2C_2O_4$/100 ml., no difficulty was encountered in obtaining a crystalline precipitate of $Ce_2(C_2O_4)_3$ at *p*H 6. In 2 hr. 98% of the oxalate was precipitated. This result indicates that an aqueous solution of oxalate of the strength which Suzuki [1934] supposed to be present in blood filtrates yields an easily visible crystalline precipitate, suitable for analysis. However, when the precipitation was attempted under the conditions set out by Izumi [1933], viz. at *p*H 2·3 (colorimetrically, thymol blue) and in the presence of $CCl_3.COOH$, no precipitate could be obtained. In these experiments the number of drops of $Ce_2(SO_4)_3$ was varied from 1 to 4 (0·04–0·16 ml.) and the solutions were left overnight. Since oxalate is not precipitated from aqueous solution under these conditions it is improbable that a precipitate of $Ce_2(C_2O_4)_3$ would separate from a blood filtrate. Experiments showed that in some cases a small amorphous precipitate did appear, but in no case could oxalate be recovered when the precipitate from 10 ml. of blood was subjected to the process of Dodds & Gallimore [1932]. This result therefore supports the contention of Thomsen [1935].

(2) *Investigation of the precipitate obtained with lime water* [Merz & Maugeri, 1931]. If the precipitate consists of CaC_2O_4 it should be possible to convert it into $Et_2C_2O_4$ and to estimate the quantity so esterified by the procedure of Dodds & Gallimore [1932]. The 'oxalate' content of a sample of heparinized human blood was determined, and 4·55 mg./100 ml. were found. The precipitate from 20 ml. of blood (0·91 mg. 'oxalate') was dissolved in a small quantity of HCl and transferred to the Claisen flask of the apparatus used for the distillation of the ester. Only 0·092 mg. oxalate was recovered. It is probable that at least a part of this oxalate arose from the decomposition of ascorbic acid of