LXX. THE CHARACTERISTIC ABSORPTION OF ULTRA-VIOLET RADIATION BY CERTAIN PURINES.

By ENSOR ROSLYN HOLIDAY.

From the Medical Unit and Physiological Laboratories, The London Hospital.

(*Received April 18th, 1930.*)

The measurement of the absorption of ultra-violet radiations by biological substances, as effected with the spectrophotometer, has been used with some success in recent years. Using this method Smith [1928] measured the ultra-violet absorption of uric acid and pointed out the close correspondence of the absorption of an ultra-filtrate of human plasma with that of a simple solution of uric acid.

In some unpublished work, the present writer has found that ultra-filtrates of certain other body fluids and tissue extracts have markedly different absorptions from that of plasma. The substances most likely to cause this absorption would seem to be purines.

It was therefore decided to establish the characteristic absorption of these substances and so obtain a basis for future investigation, both quantitative and qualitative, of body fluids. In this paper the results of this investigation are reported.

Historical.

Soret [1883] and Hartley [1905] investigated the absorption of purines in the ultra-violet region. Later Dhéré [1909] examined purines and pyrimidines and showed that they all exhibited selective absorption. He also demonstrated that uric acid shows one absorption band in alkaline solution and two bands in acid solution. These observations were not quantitative, owing to the limitations of the methods employed.

Smith [1928], using a Hilger spectrophotometer with rotating sector, was able to measure the absorption of uric acid quantitatively, and confirmed Dhéré's observations in acid and alkaline solutions. Recently Marchlewski and Wierznchowska [1929] have investigated adenine, guanine and uric acid in the same way; they failed however to observe the effect of hydrogen ion concentration on the ultra-violet absorption spectra.

Methods.

The absorption spectra were measured with a Hilger quarter-plate spectrophotometer with rotating sector. The source of radiation was a condensed spark between tungsten-steel electrodes. A Hilger micrometer cell was used for determining whether Beer's law was followed. The thickness of the cell could be varied from 0 to 5 mm., the micrometer being graduated in 0·01 mm. and reading to 0·01 mm. with 5 % error.

Ilford process plates were used except where investigation of the spectrum of shorter wave-lengths than 2150 Å. was desired, in which case Schumann plates (Hilger) were employed.

Experimental.

Each purine was examined with the object of showing:

(1) whether Beer's law was followed;

(2) the effect of hydrogen ion concentration on the absorption spectra;

(3) the effect of chemical combination of the purine with a molecule which has no appreciable absorption at the same wave-length.

The purines were examined in as pure a state as possible. Guanine and adenine and their nucleotides were isolated from yeast nucleic acid by the method of Jones [1914]. Hypoxanthine was obtained from meat extract. The adenosine was a commercial product and two commercial samples of uric acid were investigated. The nitrogen content was taken as the standard of purity and recrystallisation repeated until the estimated content agreed within 1 % of the theoretical value.

(1) *Beer's law*[1].

The solvent in all cases was water.

The thickness of the cell was varied so that in the formula

$$E = \frac{1}{cd} \log \frac{I'}{I}$$

$c \times d$ was constant. The sector used was one of the short focus type which will not hold a cell of more than 1 cm. length. The experimental error of the spectrophotometric method is least at an ϵ value on the rotating sector of

[1] Beer's law states that the absorptive power of molecules is independent of their concentration. This can be expressed graphically by

$$\log \frac{I}{I'} \times \frac{1}{cd} = \text{constant},$$

where I is the intensity of the incident beam of monochromatic light and I' the intensity of the same light after transmission through a layer of absorbing solution of thickness d and concentration c.

The ratio $\log \frac{I}{I'}$ is expressed as ϵ (the extinction coefficient) and is given by the reading on the sector. E (the specific extinction coefficient) has been taken in this paper as being the constant in the above expression. Molecular E is this constant when $d = 1$ cm. and c is the molecular weight of the substance in grams in one litre and is of importance only for purposes of comparison, having no practical meaning.

1·0 to 1·1. This results from two opposing sources of error. The lower the ϵ value on the rotating sector the higher the percentage error, due to the error of the spectrophotometer being constant over all ranges [von Halban and Eisenbrand, 1927]. On the other hand, the higher the ϵ value on the sector the more difficult is it to set the sector accurately owing to the scale divisions being logarithmically spaced and very close at high ϵ values. A mean is struck at $\epsilon = 1{\cdot}0$–$1{\cdot}1$ where the mechanical error is low and the accurate setting of the sector relatively easy. From these considerations the weakest solution was made so that with a thickness of 1 cm. ϵ had a value of 1·1.

Five dilutions were examined ranging from the weak to a saturated solution. Owing to the slight solubility of the purines in water the range is not large, the saturated solution having about 25–40 times the concentration of the weakest solution examined.

All the purines examined follow Beer's law within the limits investigated.

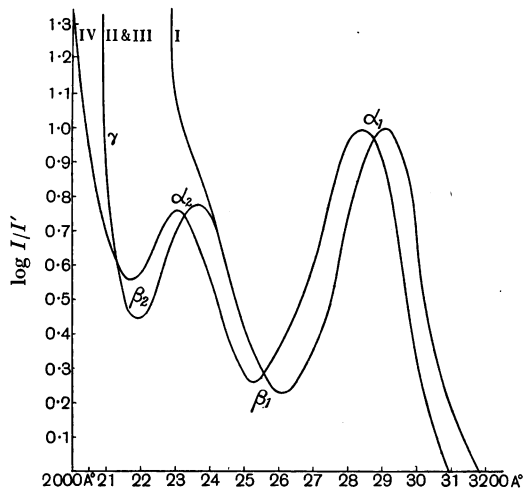

Fig. 1. Uric acid. Effect of hydrogen ion concentration on absorption curve.
I $p_H = 9{\cdot}1$. II $p_H = 7{\cdot}8$ III $p_H = 6{\cdot}8$ IV $p_H = 2{\cdot}0$

(2) *Hydrogen ion concentration.*

Owing to their general absorption at the shorter wave-lengths, buffer solutions were found unsuitable for obtaining solutions of given hydrogen ion concentration. Solutions of purines in suitable strength were made up in alkali (NaOH) and titrated with acid (HCl) to an approximate p_H. This was subsequently determined by the glass electrode method [Kerridge, 1925]. The results are shown in Tables I and II. Where the effect of hydrogen ion concentration is small, determinations at only two p_H values are given.

Absorption curves of uric acid and guanine showing the effect of hydrogen ion concentration are given in Figs. 1 and 2.

Table I.

	p_H	Molecular E α apex	Molecular E β base	Apex/base ratio α/β	Wave-length (Å.) α	β	γ
Adenine	3·0	$1·4 \times 10^5$	$0·26 \times 10^5$	5·4	2630	2300	2110
	10·0	$1·4 \times 10^5$	$0·26 \times 10^5$	5·4	2660	2380	2230
Adenine nucleotide	4·0	$1·3 \times 10^5$	$0·24 \times 10^5$	5·5	2620	2290	2110
Adenosine	8·0	$1·3 \times 10^5$	$0·24 \times 10^5$	5·5	2640	2340	2200
Hypoxanthine	3·0	$0·9 \times 10^5$	$0·15 \times 10^5$	5·6	2450	2210	2070
	7·0	$0·9 \times 10^5$	$0·15 \times 10^5$	5·6	2490	2220	2080
	11·0	$0·9 \times 10^5$	$0·15 \times 10^5$	5·6	2590	2300	2170
Caffeine	3·0	$0·8 \times 10^5$	$0·14 \times 10^5$	5·6	2740	2440	2180
	10·0	$0·8 \times 10^5$	$0·14 \times 10^5$	5·6	2710	2430	2190
Theobromine	3·0	$0·9 \times 10^5$	$0·3 \times 10^5$	3·0	2720	2410	2190
	10·0	$0·9 \times 10^5$	$0·3 \times 10^5$	3·0	2740	2480	2210
Ergothioneine	—	$1·1 \times 10^5$	—	—	2570	—	—

Table II.

	p_H	Molecular E α_1	β_1	α_2	β_2	Wave-length (Å.) α_1	β_1	α_2	β_2	γ
Uric acid	9·1	$1·0 \times 10^5$	$0·25 \times 10^5$	—	—	2920	2610	—	—	2315
	7·8	$1·0 \times 10^5$	$0·25 \times 10^5$	$0·76 \times 10^5$	$0·45 \times 10^5$	2920	2610	2360	2190	2090
	6·7	$1·0 \times 10^5$	$0·25 \times 10^5$	$0·76 \times 10^5$	$0·45 \times 10^5$	2920	2610	2360	2190	2090
	2·0	$1·0 \times 10^5$	$0·25 \times 10^5$	$0·76 \times 10^5$	$0·55 \times 10^5$	2840	2530	2300	2170	2040
Guanine	9·0	$0·63 \times 10^5$	—	—	$0·43 \times 10^5$	2760	—	—	2300	2240
	8·8	$0·63 \times 10^5$	—	—	$0·43 \times 10^5$	2760	—	—	2300	2200
	6·8	$0·63 \times 10^5$	$0·52 \times 10^5$	$0·64 \times 10^5$	$0·43 \times 10^5$	2760	2620	2500	2300	2180
	2·4	$0·63 \times 10^5$	$0·52 \times 10^5$	$0·82 \times 10^5$	$0·33 \times 10^5$	2760	2620	2500	2230	2120
	0·87	—	—	$1·09 \times 10^5$	$0·32 \times 10^5$	—	—	2480	2230	2090
Guanine nucleotide	1·0	—	—	$1·05 \times 10^5$	$0·26 \times 10^5$	—	—	2500	2230	2090

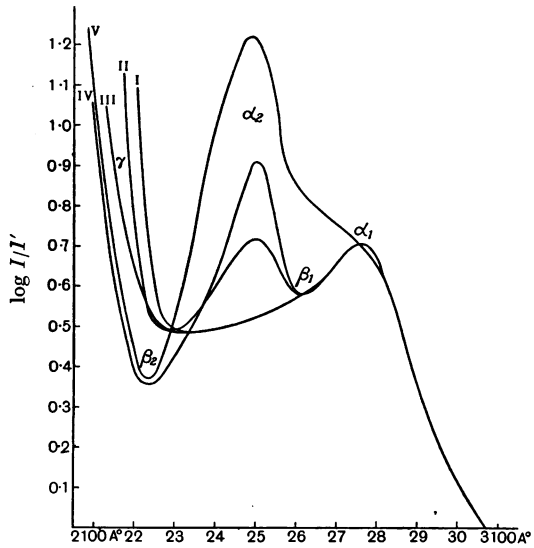

Fig. 2. Guanine. Effect of hydrogen ion concentration on absorption curve.

I $p_H = 9·0$ II $p_H = 8·8$ III $p_H = 6·8$ IV $p_H = 2·4$ V $p_H = 0·87$

Discussion of results.

It will be seen that purines can be divided into two groups: (1) those which have only one absorption band (Table I), and (2) those which have two bands in acid solution (Table II). The absorption of the first group is little influenced by increase in hydrogen ion concentration, the only effect being a slight shift of the absorption towards the shorter wave-lengths. The average shift is about 30 Å. with a change from p_H 10·0 to p_H 3·0. The exception is hypoxanthine, which shows a shift of 140 Å. over the same range of p_H. The extinction coefficient is unaffected in all cases by change of hydrogen ion concentration, which is in disagreement with Smith [1928], who found a slightly higher extinction coefficient for uric acid in alkaline than in acid solution.

The second group comprises uric acid and guanine. In the case of uric acid (Fig. 1) change of the absorption takes place in two stages: (i) between p_H 9·1–7·8 (curves I and II) and (ii) between p_H 6·8–2·0 (curves III and IV). No change occurs between p_H 7·8–6·8 (curves II and III). Two dissociation constants for uric acid [His and Paul, 1900] are given as 2×10^{-6} and $2{\cdot}0 \times 10^{-9}$ which correspond to p_H values of 5·7 and 8·7 respectively. It seems probable, therefore, that in the case of uric acid the absorption changes correspond to changes between acidic and basic forms of the molecule.

In the case of guanine the change in the absorption seems to be a gradual one throughout the range of p_H. One dissociation constant is given for guanine by Wood [1903] as $8{\cdot}4 \times 10^{-12}$, which corresponds to a p_H of 11·0. This is outside the range shown in Fig. 2, but a solution of guanine at that p_H shows the same absorption curve as I in Fig. 2. A titration curve of guanine showed no evidence of a second dissociation constant. From its constitution as a 2-amino-6-hydroxypurine one would expect guanine to have two dissociation constants. If, as has been suggested in the case of uric acid, absorption changes result from intramolecular rearrangements in the dissociation of guanine, it can be seen that the dissociation into the more basic form is spread over a wide range. This is probably the reason for the difficulty in determining the second dissociation constant.

The results show the importance of determining the hydrogen ion concentration in any spectrophotometric method of estimating or identifying purines.

It will be seen that chemical combinations have no effect on the absorption of the purines as judged by the absorption spectra of their nucleosides or nucleotides. The absorption of these substances is identical with that of the corresponding purines under the same conditions.

In general it may be said that the spectrophotometric method offers a fresh mode of attacking the problems of purine metabolism. It must be emphasised, however, that it can only be an adjunct to the usual biochemical methods of analysis and is useless by itself for investigating tissue fluids.

Even if the substances causing selective absorption in these fluids are purines, it can be seen from the tables that the absorption bands of the different members occur so close to one another that it would be impossible to identify and estimate two such occurring in roughly equivalent amounts. On the other hand, as a rapid method of estimating separate members of the purines the method is of great value, and the fact that nucleosides and nucleotides are estimated in terms of the contained purine might be made use of in estimating proportions of free and combined purine. In the case of uric acid, which Benedict [1923] states occurs in ox-blood combined as a riboside, the spectrophotometric method has shown, in preliminary experiments by the writer, that, by a simple method of difference, an estimate of the amount of combined uric acid can be obtained. The chemical methods only estimate free uric acid and the spectrophotometric method total uric acid, free and combined.

The chemical methods are few and of doubtful reliability. The results for uric acid in normal human blood are probably of fair accuracy, but this does not apply to animal blood [Bulmer, Hunter and Eagles, 1925]. Buell and Perkins [1928] have developed a nephelometric method for determination of adenine nucleotide in blood but, apart from these, no methods for estimating purines in small amounts of blood are available. It therefore seems reasonable to hope that the spectrophotometric method, if used with proper precautions, may be of value in investigations of purine metabolism.

It was thought of interest to include observations on ergothioneine. This compound, thiolhistidinebetaine, exists in blood and interferes with colorimetric determinations of uric acid. It was isolated from pig's blood by Hunter and Eagles [1925]. It shows selective absorption very similar to that of purines and the molecular extinction coefficient is of the same order. The interest of its absorption spectrum lies, however, in the fact that it is completely transparent to rays of short wave-length as far as 1900 Å.

Summary.

1. The ultra-violet absorption spectra of the following purines has been determined: uric acid, guanine, adenine, hypoxanthine, caffeine and theobromine.

2. Beer's law is followed by all.

3. The effect of hydrogen ion concentration on the absorption spectra has been measured and its importance emphasised.

4. Combination of a purine in the form of a nucleoside or nucleotide does not alter the absorption.

5. The ultra-violet absorption spectrum of ergothioneine has also been determined.

The writer wishes to express his indebtedness to Dr J. R. Marrack for the hydrogen ion determinations, to Dr H. D. Kay for the adenine and guanine

nucleotides, to Dr Eagles for the sample of ergothioneine and to Mr Campbell Smith for many valuable suggestions.

This work was done during the tenure of a scholarship given by the Worshipful Company of Grocers.

REFERENCES.

Benedict (1923). *J. Biol. Chem.* **51**, 187.
Buell and Perkins (1928). *J. Biol. Chem.* **76**, 95.
Bulmer, Hunter and Eagles (1925). *J. Biol. Chem.* **63**, 15.
Von Halban and Eisenbrand (1927). *Proc. Roy. Soc. Lond.* A **116**, 153.
Hartley (1905). *J. Chem. Soc.* **87**, 1796.
Dhéré (1909). L'absorption des rayons ultra-violets par les albuminoïdes, les proteïdes et leurs dérivés (Fribourg).
His and Paul (1900). *Z. physiol. Chem.* **31**, 1.
Hunter and Eagles (1925). *J. Biol. Chem.* **65**, 623.
Jones (1914). Nucleic acids. (Longmans Green and Co.)
Kerridge (1925). *Biochem. J.* **19**, 611.
Marchlewski and Wierznchowska (1929). *Bull. Acad. Polonaise*, 1 A, 165.
Smith (1928). *Biochem. J.* **22**, 1499.
Soret (1883). *Arch. Sci. Phys. Nat.* 3^{e} *période*, **10**, 429.
Wood (1903). *J. Chem. Soc.* **83**, 568.