molecule, and it is probable that they differ in their action on complement.

The combination of *H*-sulphone (HO_2) and divinyl sulphone with proteins is mainly concerned with a reaction of the sulphone with the free amino-groups of the protein, and it is possible that inactivation of complement by these two reagents is effected by this means. In the case of *H*, however, the reaction with proteins is apparently largely concerned with the carboxyl and iminazole groups (Ball, 1942). The rate of combination of protein with *H* is somewhat slower than that with HO_2 (Banks *et al.* 1946*b*), and it may seem rather surprising that complete inactivation of complement was effected, under the conditions of the experiments described in this paper, more rapidly with *H* than with an equivalent amount of HO_2 . Complement inactivation might, however, be the result of the action of *H* or HO_2 on a few essential groups in one or more of the components of complement, and the rate of inactivation may bear no relationship to the total amount of *H* or HO_2 which has combined with the serum proteins.

Another point which we intend to investigate further is the significance of the SH groups of the serum proteins as far as complement activity is concerned. *H*, HO_2 and divinyl sulphone all readily react with SH groups, and a reaction of this type might account for part at least of their inactivating

action on complement. In this connexion it is of interest to note that Ecker, Pillemer, Martiensen & Wertheimer (1938) have suggested that complement may possess SH groups, and that inactivation by oxidation might be due to their conversion to disulphide ($-S-S-$) groups.

SUMMARY

1. Small amounts of mustard gas (*H*) rapidly inactivate complement. The inactivation is not due to pH changes.
2. The inactivation appears to affect all the components of the complement. There is some indication, however, that the relatively heat-labile components (C'1 and C'2) are more rapidly inactivated than the other two components.
3. Divinyl sulphone similarly inactivates complement, but even more rapidly than does *H*.
4. *H*-Sulphone ($\beta\beta'$ -dichlorodiethyl sulphone) inactivates complement, but relatively slowly compared with *H* and divinyl sulphone.

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The Polarographic Estimation of Steroid Hormones

3. COMPARISON OF NORMAL VALUES FOR URINARY 17-KETOSTEROIDS BY POLAROGRAPHIC AND COLORIMETRIC METHODS

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In the work of Wolfe, Hershberg & Fieser (1940) referred to in a previous communication (Barnett, Henly & Morris, 1946), a comparison was made between the polarographic and the Zimmermann (1935, 1936) methods for the estimation of neutral urinary 17-ketosteroids. Most of the colorimetric assays (for

example, those supplied by Dr Talbot) were significantly higher than the polarographic results, whilst those obtained by another group were lower. Wolfe *et al.* consider that this was due to the high correction factor applied by this group to their Zimmermann values. They do not give any figures for the

24 hr. output of neutral 17-ketosteroids. We have therefore carried out a series of duplicate estimations of the 24-hourly output of neutral 17-ketosteroids on eight young medical students and twenty young nurses, and compared the results obtained by the polarographic and colorimetric (Zimmermann-Callow) methods (Callow, Callow & Emmens, 1938).

We have also compared the results obtained from the twenty women with the values already obtained by us (Barnett *et al.* 1946) on twenty-four men, in order to assess the significance of the difference between the average values for men and for women.

EXPERIMENTAL

Eight medical students (aged 20–22 years), and twenty nurses (aged 18–37 years) were selected for this study; the method of collection and the importance of accurate collection of specimens were explained to them very carefully. Extracts from each specimen were made in duplicate (in the men's group by two different workers; in the women's group by the same person). The duplicates were examined polarographically* as described by Barnett *et al.* (1946); the same duplicate extracts were also assayed by the Zimmermann-Callow method by one of us (F. L. W.) who had had considerable experience of this technique.

RESULTS

The results are summarized in Tables 1 (males) and 2 (females). Both methods agree in giving a lower figure for the women's ketosteroid output than for the men's. Fisher's '*t*' test applied to the figures for eight men and twenty women give a value of $t = 3.54$ (polarographic method) and $t = 2.87$ (colorimetric method); for 26 degrees of freedom this gives values for P of < 0.01 for both methods, i.e. a probability of less than 1% that the difference in the mean value for men and for women is due to chance.

It would appear that in general the colorimetric method gives higher results than the polarographic method. The results by both methods were referred to the same standard specimen of *iso*-dehydroandrosterone.

In the case of the duplicate values obtained on the males, the colorimetric estimations (which were all done by one person) gave a lower variation among duplicates than the polarographic estimation (when the two sets of duplicates were carried through by two different workers). On the other hand, in the case of the female values, the duplicates were done in

each method by one person, and here the variation among duplicates was very considerably less by the polarographic method than by the Zimmermann method. It is interesting to note the extremely high reproducibility of the polarographic results here (since the deviation among duplicates is entirely a measure of variation due to technique and not to biological variation).

The overall standard deviation of the male and female figures (means of duplicates) shows again a lower variation in both sets by the polarographic than by the colorimetric method; as the extracts examined were the same for both methods, this would again indicate a lower variation due to the errors of technique in the polarographic than in the colorimetric method.

Finally, a comparison of the values obtained for the twenty women quoted here, with those obtained for twenty-four young men (Barnett *et al.* 1946), both by the polarographic method, shows by application of Fisher's *t* test, a significant difference between the mean value for the male and for the female output ($t = 4.136$ which for 42 degrees of freedom indicates $P \ll 0.01$, i.e. the probability of this result occurring by chance is less than 1%. The distribution of the results about the mean was normal in both sets).

DISCUSSION

For the purpose of comparison, the various data on the excretion of neutral 17-ketosteroids by normal men and women, as estimated by different workers are collected in Table 3. To the best of our knowledge, the table includes most of the figures published since 1939 (in which the number of cases is not less than eight). We have calculated the average value, the range of normal values and the standard deviation from these sets of figures, in order to be able to compare them readily. On examination of Table 3, several facts are apparent: in the first place, there is very considerable disagreement as to the average normal value and the range of normal values both in men and women. It should be borne in mind that this variation (as typified, for example, by the standard deviation) is the sum of two distinct factors, viz. the biological variation among individuals, and the error of the method. However, if large enough groups are taken, the differences in the mean values are most probably due to differences in technique.

Secondly, the values reported by us in this paper are considerably higher than those reported elsewhere. This may possibly be due to the fact that the subjects in our investigation were all young, while in most of the other groups quoted the age groups were considerably wider, there being some evidence that ketosteroid output decreases with increasing

* It has been noted that certain samples of A.R. sodium chloride, used in the preparation of the base solution for the polarographic method (Barnett *et al.* 1946), contain an impurity which produces a small polarographic wave at about -1.1 V. This wave interferes with the 17-ketosteroid wave, so that care must be taken in the selection of sodium chloride for polarographic use.

Table 1. *The excretion of 17-ketosteroids in eight normal young men, measured polarographically and colorimetrically*

Exp. no. index	Age (years)	24-hourly urinary vol. (ml.)	Polarographic method (mg./24 hr.)			Zimmermann method (mg./24 hr.)		
			Set A (AAH)	Set B (JB)	Mean	Set A	Set B	Mean
M20	20	1910	23.4	18.6	21.0	26.2	28.8	27.5
M21	20	900	15.2	18.0	16.6	20.4	17.3	18.8
M22	20	1030	13.9	13.6	13.7	13.2	13.6	13.4
M23	21	1500	20.2	18.7	19.5	21.0	20.1	20.6
M24	20	1570	19.6	17.5	18.5	19.5	17.6	18.6
M25	22	2070	17.6	17.6	17.6	18.2	20.3	19.2
M26	22	2090	17.7	12.5	15.1	17.6	14.5	16.1
M27	21	1700	13.6	11.9	12.8	12.4	12.9	12.7
Mean of both sets A and B				16.8			18.4	
Standard deviation of means of both sets				2.94			4.65	
Standard deviation among duplicates				2.05			1.49	
Range (mean $\pm 2\sigma$)				10.9-22.7			9.1-27.7	
Correlation coefficient between polarographic and colorimetric values = 0.87.								

Table 2. *The excretion of 17-ketosteroids in twenty normal young women, measured polarographically and colorimetrically*

Exp. no. index	Age (years)	24-hourly urinary vol. (ml.)	Polarographic method (mg./24 hr.)			Zimmermann method (mg./24 hr.)		
			Set A	Set B	Mean	Set A	Set B	Mean
W30	29	550	9.42	8.8	9.1	10.0	11.7	10.9
W31	20	1700	16.5	17.5	17.0	22.3	22.6	22.4
W32	37	1880	17.2	16.6	16.9	21.2	21.6	21.4
W33	24	1210	10.7	10.4	10.6	11.7	12.0	11.9
W34	23	1400	7.6	7.2	7.4	8.6	7.7	8.2
W35	20	1410	15.3	15.3	15.3	18.0	14.2	16.1
W36	19	1420	15.8	15.4	15.6	18.9	20.0	19.5
W37	28	1100	5.15	5.35	5.3	7.2	7.0	7.1
W38	22	1510	6.9	7.3	7.1	10.2	10.4	10.3
W39	20	1690	9.65	9.65	9.7	15.3	15.0	15.2
W40	28	1470	12.6	12.6	12.6	15.0	15.4	15.2
W41	23	2640	10.6	10.6	10.6	6.0	8.9	7.5
W42	22	1820	17.1	16.6	16.9	18.0	13.7	15.8
W43	24	1740	9.4	8.9	9.2	6.2	6.2	6.2
W44	21	1890	17.8	18.4	18.1	15.1	14.6	14.9
W45	21	850	10.2	10.0	10.1	9.5	9.5	9.5
W46	18	1620	8.3	8.8	8.6	7.3	8.5	7.9
W47	19	1500	13.7	13.3	13.5	12.8	15.6	14.2
W49	20	1720	13.25	13.25	13.3	16.4	17.6	17.0
W50	21	2320	11.9	12.6	12.3	13.9	11.3	12.7
Mean of both sets A and B				11.9			13.2	
Standard deviation of means of both sets				3.76			4.75	
Standard deviation among duplicates				0.32			1.27	
Range (mean $\pm 2\sigma$)				4.3-19.5			3.8-22.6	
Correlation coefficient between polarographic and colorimetric values = 0.83.								

age. However, it is doubtful whether this can account for the low averages (viz. 9 mg.) reported by some workers. In this connexion it might be stressed again that by obtaining the co-operation of students and nurses, we have reasonable confidence that the collection of 24 hr. specimens was carried out very accurately.

The fact that the Zimmermann method gives results which appear to be systematically higher than the polarographic method (Tables 1 and 2) may

possibly be accounted for by the fact that the former reagent is known to give a colour with certain substances other than 17-ketosteroids (e.g. with cholestanone, cholestenone and other 3-ketosteroids. On the other hand, the polarographic method gives no wave with saturated 3-ketosteroids, while with Δ -4:5-3-ketosteroids it gives a wave at a different half-wave potential from that of the 17-ketosteroids, this wave being largely eliminated under the conditions of our procedure.

Table 3. *The excretion of neutral 17-ketosteroids by normal men and women as reported by various workers since 1939*

Authors	No. of cases	Sex	Average keto-steroid output (mg./24 hr.)	Standard deviation (mg.)	Coefficient of variation (%)	Range (mean $\pm 2\sigma$) (mg./24 hr.)	Details of method, etc.
Callow, Callow, Emmens & Stroud (1939)	10	M.	9.05	2.5	28	4-14	Callow modification of Zimmermann method ⁽¹⁾
Chou & Wang (1939)	16	M.	9.5	3.4	36	2.7-16.3	Colorimetric method other than Zimmermann ⁽³⁾
Baummann & Metzger (1940)	11	M.	15.1	3.0	19	9-21	Zimmermann-Callow method ⁽³⁾
Fraser, Forbes, Albright, Sulko-witch & Reifenstein (1941)	9	M.	13.7	4.5	33	4.7-22.7	Zimmermann-Callow method: 16 hr. night sample taken, $\times \frac{2}{3}$ for 24 hr. output ⁽⁴⁾
Cuyler, Hamblen, Baptist & Salmon (1942)	10	M.	12.5	6.3	51	0-25	17-ketosteroids estimated by method of Oesting (1937, 1938) after extraction by method of Cuyler & Baptist (1941) ⁽⁶⁾
Patterson, McPhee & Greenwood (1942)	9	M.	13.3	4.0	31	5.3-21.3	Zimmermann-Callow method ⁽⁶⁾
Pincus & Hoagland (1943)	16	M.	9.2	1.8	19	5.6-12.8	Zimmermann-Callow method ⁽⁷⁾
Salter, Cahen & Sappington (1946)	14	M.	14.2	4.0	28	6.2-22.2	Pincus (1943)
Ibid.	14	M.	15.9	3.7	23	8.5-23.3	Modified Zimmermann } Colorimetric methods on (Cahen & Salter, 1944) } same extracts ⁽⁸⁾
Barnett, Henly & Morris (1946)	24	M.	15.7	2.4	15	10.9-20.5	Polarographic method ⁽⁹⁾
Barnett, Henly, Morris & Warren (this paper)	8	M.	16.8	2.9	17	11.0-22.6	Polarographic } Estimation on duplicates of same eight cases ⁽⁹⁾
Fraser, Forbes, Albright, Sulko-witch & Reifenstein (1941)	8	M.	18.4	4.6	25	9.2-27.6	Zimmermann-Callow
Patterson, McPhee & Greenwood (1942)	14	F.	9.0	2.6	29	3.8-14.2	Zimmermann-Callow method: 16 hr. night sample taken, $\times \frac{2}{3}$ for 24 hr. output ⁽⁴⁾
Salter, Cahen & Sappington (1946)	12	F.	7.4	3.0	40	1.4-13.4	Zimmermann-Callow method ⁽⁶⁾
Ibid.	14	F.	7.9	2.9	37	2.1-13.7	Pincus (1943)
Barnett, Henly, Morris & Warren (this paper)	14	F.	8.0	2.5	32	3.0-13.0	Modified Zimmermann } Colorimetric methods on (Cahen & Salter, 1944) } same extracts ⁽¹⁰⁾
Ibid.	20	F.	11.9	3.8	32	4.3-19.5	Polarographic } Estimation on duplicates of same twenty cases ⁽¹¹⁾
Ibid.	20	F.	13.2	4.7	35	3.8-22.6	Zimmermann-Callow

(1) Values are each the mean of several estimations on different collections. Subjects were patients with no sexual dysfunction.

(2) Each value is the mean of three estimations on different 24 hr. collections. Subjects were aged 21-28 years.

(3) Extracts purified by adsorption on charcoal and elution with ether. Subjects were aged 17-30 years (one man aged 49 has been omitted from this calculation).

(4) Each value is the mean of several estimations.

(5) Subjects were normal males selected on the basis of normal seminal values (4-7 consecutive 24 hr. samples taken for each estimation). Ages were 20-37 years (one man aged 48 omitted from this calculation).

(6) Mainly single determinations.

(7) 24 hr. output calculated by expressing the value for a day specimen (of a few hours only) in terms of the night value. Each value is mean of three estimations. Subjects were young students and pilots.

(8) Mainly single determinations. Subjects were aged 20-58 years.

(9) Each value is mean of duplicate determinations on same specimen. Subjects were aged 19-22 years.

(10) Mainly single determinations. Subjects were aged 22-54 years.

(11) Subjects were aged 18-37 years.

SUMMARY

1. The excretion of neutral 17-ketosteroids in eight normal young men and twenty normal young women has been estimated in duplicate both by the polarographic method and by the Zimmermann-Callow colorimetric technique.

2. The colorimetric method gave somewhat higher results than the polarographic method and its standard deviation was also higher. There is some

evidence that the polarographic method shows a lower variation due to the errors of technique than the colorimetric method.

3. These values, compared with available data published by other workers on normal 17-ketosteroid excretion, would seem to be higher than any other figures published.

The authors would like to express their thanks to the subjects who co-operated in the collection of 24 hr. specimens.

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An Analysis of the Products Formed During the Hydrolysis of Hyaluronate by Enzymes and Acids, with Observations on the Nature of the Amino-sugar Released from the Polysaccharide

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It has been shown (Rogers, 1945, 1946) that after the hydrolysis at pH 7.0 of purified potassium hyaluronate by either partially purified or crude hyaluronidase from bull testis, substances, non-diffusible through cellophane, remain in the hydrolysate which can adaptively increase hyaluronidase production by multiplying streptococci. No such substances remain after hydrolysis of the polysaccharide at pH 7.0 by hyaluronidase produced by either *Cl. welchii* or streptococci.

In the present paper these non-diffusible constituents of the hydrolysates are shown to differ chemically according to the source of the enzyme used for hydrolysis. This result indicates the probability that more than one enzyme is involved in the complete hydrolysis of hyaluronate. A similar conclusion had been reached previously by several

authors (Madinaveitia & Quibell, 1940; East, Madinaveitia & Todd, 1941; Meyer, Chaffee, Hobby & Dawson, 1941; Hahn, 1945*a*). Since the work described in the present paper was completed, however, definite evidence has been published (Hahn, 1945*b*, 1946*a*, *b*) which indicates the presence in crude preparations from mammalian testis of two enzymes which, when acting at pH 4.6, can together degrade hyaluronate to monosaccharides. The pH of 4.6 has been shown to be near the optimum for the activity of testicular hyaluronidase when concentrated buffers are used (Madinaveitia & Quibell, 1940; Meyer *et al.* 1941; McClean, 1943). Hydrolysis was carried out at pH 7.0 and in dilute buffer and salt solutions in the present work, because these were the conditions used in the biological studies (Rogers, 1945, 1946) and have been shown (McClean, 1943)