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Quantitative Colorimetrical Determination of Certain Sex Hormones belonging to the Steroid Group

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The biological methods for the determination of sex hormones are laborious and not very accurate. Of chemical methods, gravimetric ones are impossible, and colorimetrical ones are not very satisfactory, particulary in that they do not distinguish between substances closely related chemically. Therefore this work is presented.

The oldest colour reaction is that with conc. H_2SO_4, which is unspecific. It was first communicated by Wieland, Straub and Dorfmüller [1]. The blue fluorescence is given by a multitude of organic substances. Upon diluting with water a reddish, unstable colour is formed that is made more stable by addition of phenol or β-naphtol. The colorimetrical method of Kober [2] is built on this observation. Schmulowitz, Schwenk-Hildebrandt and Marx [3-5] have communicated experiments where they try to bring about a coupling between the hydroxyl group in the hormones of the oestrone family and the diazo group. Pincus, Wheeler, Young and Zahl [6] describe a colour reaction analogous to the cholesterol reaction with benzoyl chloride.

Scherrer [7] stated that sterols dissolved in conc. H_2SO_4 and covered with a layer of benzaldehyde will develop coloured rings in the interface. Oyama [8] described a microdetermination of cholesterol with salizylaldehyde and conc. H_2SO_4. The author, having had no knowledge of this work, made som preliminary tests of his own, and found that the steroid hormones dissolved in conc. H_2SO_4 will develop strong but unstable colours after treatment with benzaldehyde. The use of benzaldehyde in the presence of conc. H_2SO_4 is not to be recommended because of the lability of the benzaldehyde; but if *p*-dimethylaminobenzaldehyde is used instead, a distinct, stable and reproducible colour results. The fact that *p*-dimethylaminobenzaldehyde (reagent P) gives colours with a series of phenols was reported by Joachimowitz [9] and used in an investi-

gation on plant cells. The colours produced with the reagent P and these hormones can sometimes be considerably intensified by addition of water. The colour thus obtained is stable for hours, its intensity is proportional to the amount of hormone present (*e. g.* oestrone), and can easily be measured in the step-photometer (»stufo»). Instead of reagent P, *m*-Nitrobenzaldehyde (reagent M) can be used. *p*-Nitrobenzaldehyde gives weaker colours. *o*-Nitrobenzaldehyde gives no colour at all. Acetaldehyde as well as formaldehyde is usuable but these offer no advantage compared with the reagent P.

METHOD

A standard solution is made from the pure substance and the desired amount transferred to a test tube by a pipette. The solvent ($CHCl_3$) is evaporated on the boiling water-bath. After cooling, 0.2 ml of 1 % solution of reagent P or M in $CHCl_3$ is added and the solvent removed in vacuo at about 60°. When reagent P is used, the temperature must never be allowed to exceed 70° since melting of the reagent P (m. p. 73°) will lead to completely irregular blankvalues. Reagent P that has not been melted will always give reproducible blanks of low or neglegible value, depending on the age of the reagent P solution. By using reagent M all these precautionary measures are unnecessary since even after prolonged heating of reagent M (m. p. 58°) at 100° the blanks are always zero. — To the residue in the test tube 1.2 ml conc. H_2SO_4 (A. R.) is added and with oestrone, oestradiol, equilin and androsterone a cherry red colour will develop where the H_2SO_4 and the residue touch. This colour changes to red-orange after heating for 1 ½—2 min in the boiling water-bath. Oestradiol will then give a brown-orange tint. After completion of the heating the test tube is cooled under the tap, 0.8 ml water is added and the tube is vigorously and continously shaken in the stream of water. Thorough cooling is essential as overheating of the mixture will result in a variation in colour. The tube is now heated for 5 min in the boiling water-bath and after cooling under the tap ready for reading in the »stufo» with filter S 53. The compensation-cell is filled with the blank, or with water when reagent M has been used. The colour remains constant at least one hour and is permanganateviolet for the said hormones. The time given for the various steps is optimal and can easily be kept by aid of an alarm clock. The ratio H_2SO_4/H_2O is optimal. The colours given by the examined substances can be seen from Tables 1, 4 and 5. The sensitivity of reagent M is somewhat less than that of the reagent P. Beer's law is valid within wide ranges. For more than 250 μg oestrone the amount taken of reagent P or M must be increased. Insufficient quantities of the reagents can be recognized by change of colour compared with the colour when sufficient aldehyde has been used. Regarding the validity of Beer's law one is referred to Tables 2 and 3. Progesterone does not react with the reagent P or M. Pregnandiol should not be determined with the reagent P or M. The substance reacts but the results show poor conformity. Oestriol and equilinin were not available during the war. — The colour given by equilin is visually very similiar to that from oestrone, but maximal extinction is at S 57. — Phenols must be removed before determination as they also react with both reagent P and M.

The simultaneous determination of oestrone, oestradiol and equilin gives simple additive values. Instead of 0.2 ml of 1 % solution of reagent P or M, however 0.4 ml must be taken. For the determination of 50 μg oestrone together with 50 μg oestradiol

Table 1. Colours on reaction with reagent P and conc. H_2SO_4 + 50 µg hormone. a. undiluted b. diluted with 0.8 ml H_2O.

	a.	b.
1. Oestrone	red-orange	permanganate-violet
2. Oestradiol	brown-orange	red-brown
3. Equilin	red-orange	blue-violet
4. Androsterone	yellow-orange	brownish-permanganate
5. Testosterone	light-orange	yellow-brown
6. Corticosterone	light-yellow	brown-orange
7. Cholesterol	orange	red-orange
8. Pregnandiol	deep-orange	reddish-brown
9. Progesterone	colourless	faintly-orange

Table 2. Validity of Beer's law on reaction with reagent P. Final vol. 2 ml. 2.5 mm cell.

		Quantities in µg							
Substance	Filter	5	10	25	50	75	100	125	250
Oestrone	S 53	0.16	0.28	0.59	1.15	1.65	2.28*	2.87*	5.50*
Oestradiol	S 53		0.14**		0.68		1.36		
Equilin	S 57				0.78		1.58		
Testosterone	S 47				0.40		0.83		
Androsterone	S 53				0.36		0.80		
Corticosterone	S 47				0.30		0.60		
Cholesterol	S 47				0.16		0.36		

Validity of Beer's law on reaction with reagent P. Final vol. 1 ml. 10 mm microcell. Filter S 53. 0.6 ml H_2SO_4 diluted with 0.4 ml H_2O.

Substance	Quantities in µg					
	0.25	0.50	0.75	1.25	2.50	5.00
Oestrone	0.04	0.09	0.15	0.25	0.49	0.96

Table 3. Vality of Beer's law on reaction with reagent M. Final vol. 2 ml. 2.5 mm cell.

Substance	Filter	Quantities in µg		
		25	50	100
Oestrone	S 53	—	0.90	1.80
Oestradiol	S 53	0.27	0.53	1.03
Equilin	S 57	0.28	0.56	1.12
Testosterone	S 47	—	0.40	0.83
Androsterone	S 53	—	0.25	0.50
Corticosterone	S 47	—	0.30	0.55
Cholesterol	S 43	—	0.23	0.48

* Reduced from measurements in 1 mm cell.

** » » » » 5 » »

0.4 ml must be taken even if 0.2 ml is sufficient for 250 μg oestrone or oestradiol singly (Tables 6—8). The colours developed with the reagent P or M can all be destroyd by H_2O_2. With reagent P in this way were found yellow tints and the colour can be referred to the oxydation of reagent P. Reagent M will give colourless solutions. — In urine extracts the accompanying substances also react with conc. H_2SO_4 forming dark shades that disappear with H_2O_2 too.

Preparation of the solution of the reagent P and M

In all experiments solutions of 1 g reagent P or M in $CHCl_3$ were used. At room-temperature and even when kept in the dark the $CHCl_3$-solution of the reagent P will quickly turn rose-coloured and show an acid reaction. Storing at 2° will nearly suppress this process and solutions so stored will give neglegible values for the blanks for a considerable time. Ether can be substituted for $CHCl_3$; but before making up the solution it must be distilled over $SnCl_2$ because the smallest amount of peroxide will interfere with the reaction (colour as well as intensity will change). Carbontetrachloride is unsuitable

Table 4. Extinction on determination of 50 μg hormone with reagent P and conc. H_2SO_4. Final vol. 2 ml. Reading with 2.5 mm cell.

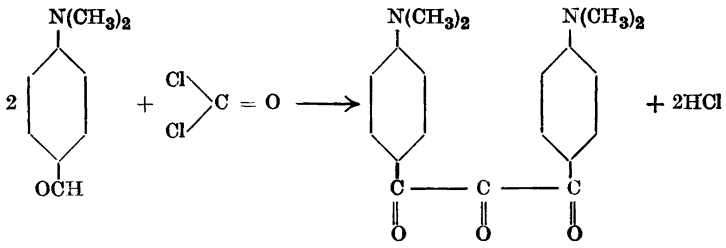
	Filters									
Substance	S 43	S 47	S 50	S 53	S 57	S 61	S 66.6	S 72	S 75	L II
Oestrone	0.22	0.37	0.73	1.15	0.33	0.22	0.11	0.00	0.00	1.00
Oestradiol	0.28	0.31	0.45	0.68	0.28	0.16	0.08	0.00	0.00	0.61
Equilin	0.29	0.40	0.44	0.68	0.78	0.58	0.15	0.03	0.00	0.62
Progesterone*	0.05	0.05	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.05
Testosterone	0.37	0.40	0.35	0.15	0.10	0.04	0.01	0.00	0.00	0.14
Androsterone ..	0.19	0.26	0.31	0.36	0.23	0.13	0.04	0.00	0.00	0.35
Pregnandiol	0.34	0.39	0.38	0.37	0.19	0.06	0.00	0.00	0.00	0.34
Corticosterone ..	0.23	0.30	0.26	0.16	0.12	0.08	0.03	0.00	0.00	0.17
Cholesterol	0.16	0.16	0.15	0.13	0.08	0.00	0.00	0.00	0.00	0.14

Table 5. Extinction on determination of 50 μg hormone with reagent M and conc. H_2SO_4. Final vol. 2 ml. Reading with 2.5 mm cell.

	Filters									
Substance	S 43	S 47	S 50	S 53	S 57	S 61	S 66.6	S 72	S 75	L II
Oestrone	0.23	0.32	0.52	0.90	0.42	0.31	0.19	0.07	0.06	0.86
Oestradiol	0.26	0.28	0.39	0.52	0.27	0.19	0.11	0.05	0.03	0.48
Equilin	0.23	0.31	0.36	0.53	0.56	0.40	0.13	0.03	0.03	0.50
Progesterone*	0.13	0.14	0.14	0.08	0.06	0.04	0.03	0.00	0.00	0.08
Testosterone	0.35	0.42	0.36	0.23	0.14	0.05	0.03	0.00	0.00	0.20
Androsterone ..	0.24	0.26	0.26	0.25	0.18	0.08	0.05	0.03	0.03	0.24
Pregnandiol	0.26	0.25	0.26	0.22	0.15	0.07	0.03	0.02	0.01	0.18
Corticosterone ..	0.25	0.32	0.24	0.10	0.09	0.04	0.00	0.00	0.00	0.13
Cholesterol	0.27	0.27	0.25	0.22	0.15	0.10	0.08	0.04	0.04	0.20

* For 100 μg progesterone the determination gives the same values.

as a solvent; a solution of reagent P quickly becomes cloudy and strongly acid. After some time a deep yellow precipitate is formed. When the solution in CCl_4 is exposed to the light from a mercury lamp, a yellow precipitate of very fine crystals is formed at once. This substance is propably 4-methyldiaminodiphenyltriketone following the reaction



Fosgen seems to be formed extremely rapidly under the influence of mercury-light in the presence of *p*-dimethylaminobenzaldehyd (acceptor-effect). There was used a »Philora» »HPW 125 W» *with* a reflector S.K.215/02 — Philiray. Without the reflector the reaction goes quite slowly. — The solution of P in $CHCl_3$ does not behave in this way; it becomes at once rose-coloured and shows an acid reaction.

Contrary to the reagent P, there is no difficulty in storing the solution of reagent M in $CHCl_3$ for months at 20°—25°. It remains stable and gives zero »blanks». Rapid, roughly quantitative tests can be carried out by adding a few crystals of reagent M and the conc. H_3SO_4 to the substance.

Table 6. Simultaneous determination of oestrone, oestradiol and equilin with reagent P. 0.4 ml of 1 % solution of reagent P. Final vol. 2 ml. Reading with 2.5 mm cell. 50 µg of each substance.

	Filters					
Substances	S 53	S 50	S 47	S 43	L II	
Oestrone + oestradiol ..	1.80	0.98	0.66	0.45	1.60	found
	1.79	1.08	0.68	0.50		calculated
Oestrone + equilin	1.66	1.04	0.72	0.45	1.10	found
	1.78	1.17	0.77	0.51	1.11	calculated
Oestradiol + equilin	1.25	0.86	0.68	0.54		found
	1.35	0.89	1.71	0.57		calculated
Oestron + oestradiol + equilin * ..	1.20	0.78	0.58	0.42		found
..	1.25	0.81	0.53	0.40		calculated

The reading with S 53 and S 50 is difficult as equality of shades and equal brightness rarely will be found. The reading is taken for equal brightness.

* 25 µg of each of the 3 substances.

Table 7. Determination of 250 µg oestrone with different quantities of reagent P. Final vol. 2 ml. Reading with 1 mm cell.

	Filters				
	S 53	S 50	S 47	S 43	
Extinction	1.44	1.13	1.08	0.54	0.1 ml of 1 % solution of reagent P
»	2.20	1.18	0.84	0.44	0.2 » » 1 » » » »
»	2.20	1.14	0.80	0.44	0.3 » » 1 » » » »
»	2.20	1.35	1.04	0.53	0.4 » » 1 » » » »

Table 8. Simultaneous determination of oestrone, oestradiol and equilin with reagent M. 0.4 ml of 1 % solution of reagent M. Final vol. 2 ml. Reading with 2.5 mm cell. 50 µg of each substance.

	Filters					
Substance	S 53	S 50	S 47	S 43	L II	
Oestron + oestradiol	1.47	0.87	0.60	0.45	1.34	found
	1.42	0.93	0.60	0.49	1.35	calculated
Oestrone + equilin	1.43	0.88	0.65	0.46	1.36	found
	1.43	0.90	0.63	0.46	1.36	calculated
Oestrone + equilin	1.09	0.73	0.61	0.53	1.00	found
	1.05	0.75	0.59	0.49	0.97	calculated
Oestrone + oestradiol + equilin *	0.98	0.68	0.48	0.38	0.87	found
	0.98	0.63	0.46	0.36	0.90	calculated

THE REACTION WITH SALIZYLALDEHYDE

Salizylaldehyde gives a colour with conc. H_2SO_4 and higher ketones. The reaction was first recommended by Täufel and Thaler [10] for the determination of the »ketone-rancidity». Under certain circumstances various sex hormones can be quantitatively determined with salizylaldehyde, conc. H_2SO_4 and glacial acetic acid. The order of mixing the reagents must strictly be adhered to.

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* 25 µg of each of the 3 substances. There are the same difficulties for reading with filters S 53 and S 50 as mentioned in Table 6.

Method

The substance to be examined is dissolved in 0.05 ml $CHCl_3$, and 2.4 ml glacial acetic acid (gl. a. a.) containing 2 % reagent S (salizylaldehyde) is added. It is first necessary to dissolve it in $CHCl_3$ because it is uncertain if the substance is completely soluble in gl. a. a. Then follows the addition of 1.2 ml conc. H_2SO_4 and vigorous shaking, immersing for 5 min in boiling water, cooling in water and dilution with 3.6 ml gl. a. a. Hereby the intensity of the colours is increased and Beer's law is valid. If the dilution with gl. a. a. is not made, other shades of colours will appear and Beer's law is *not* valid. — The compensation-cell must always be filled with the blank.

Table 9. View of the colours formed on reaction with the examined substances and gl. a. a. containing reagent S + conc. H_2SO_4. Dissolving the substance in 0.05 ml $CHCl_3$ + 2.4 ml gl. a. a. containing reagent S. Adding of conc. H_2SO_4. Diluting with 3.6 ml gl. a. a.

Substance (250 µg)	Colour
Oestrone	permanganate
Oestradiol	»
Pregnandiol	brownish permanganate
Equilin	brown
Progesterone	permanganate
Testosterone	blue-green, thick red dust *
Androsterone	brownish
Cholesterol	red violet, a tinge of brown
Corticosterone	olive coloured, thick red dust *

Table 10. View of the influence of alternating the order of mixing.

a. Dissolving the substance in conc. H_2SO_4 and adding gl. a. a. containing reagent S, no further dilution with gl. a. a.

b. Dissolving the substance in conc. H_2SO_4 and adding gl. a. a. containing reagent S. Dilution later on with more gl. a. a.

Substance (250 µg)	a.	b.
Oestrone	dirty brown	red-violet
Oestradiol	deep red	dirty orange
Pregnandiol	dirty brown	» brown
Equilin	» »	blue with a tinge of red, thick red dust *
Progesterone	red violet	dirty orange
Testosterone	deep red violet	» red
Androsterone	red, changes quickly into blue	smoky violet
Cholesterol	dirty brown	» »
Corticosterone	deep red	chestnut

* Looking at the tube against a dark background gives the impression that the glass is covered with a thin layer of red dust. The same impression is given by the walls of the »stufo»-cell when the cell is placed in the instrument.

Alternation of the order of mixing: If the substance is first dissolved in 1.2 ml conc. H_2SO_4, heated for 2 min at 100° and then 2.4 ml gl. a. a. containing reagent S is added, a shortlived vivacious play of colours is seen (Table 10). On subsequent dilution with gl. a. a. colours will appear that are considerably different from those seen by the first way of mixing. These colours do not follow Beer's law. — The sensitivity of the reaction with the reagent S is considerably less than of the corresponding reactions with the reagent P and M.

The results of the investigations on the reagent S with the various substances examined will be found in Tables 9—12.

Table 11. Extinction on determination of 500 µg substance with gl. a. a. containing reagent S and conc. H_2SO_4. Final vol. 7.2 ml. Reading in 2.5 mm cell. Compensated against the blank.

Substance	Filters									
	S 43	S 47	S 50	S 53	S 57	S 61	S 66.6	S 72	S 75	L II
Oestrone	0.06	0.18	0.38	0.62	0.25	0.20	0.08	0.00	0.00	0.66
Oestradiol	0.41	0.47	0.71	1.23	0.73	0.88	0.49	0.06	0.01	1.17
Equilin	0.36	0.63	0.70	0.60	0.29	0.27	0.13	0.00	0.00	0.57
Progesterone	0.03	0.04	0.10	0.05	0.00	0.00	0.00	0.00	0.00	0.12
Testosterone	0.32	0.52	0.47	0.56	0.57	0.54	0.42	0.16	0.08	0.60
Androsterone ..	0.35	0.33	0.36	0.40	0.31	0.20	0.08	0.02	0.00	0.44
Pregnandiol	0.22	0.27	0.35	0.43	0.49	0.30	0.12	0.02	0.00	0.46
Corticosterone ..	0.35	0.54	0.48	0.51	0.55	0.42	0.33	0.13	0.07	0.52
Cholesterol	0.31	0.40	0.43	0.48	0.34	0.28	0.19	0.03	0.00	0.54

Table 12. Validity of Beer's law on reaction with gl. a. a. containing reagent S. Final vol. 7.2 ml. Reading in 2.5 mm cell.

Substance	Filter	Quantities in µg	
		250	500
Oestrone	S 53	0.31	0.62
Oestradiol	S 53	0.59	1.23
Equilin	S 50	0.36	0.70
Progesterone	S 50	0.04	0.10
Testosterone	S 53	0.27	0.56
Androsterone	S 53	0.21	0.40
Pregnandiol	S 57	0.24	0.49
Corticosterone	S 57	0.27	0.55
Cholesterol	S 53	0.25	0.48

THE REACTION WITH ANTIMONY TRICHLORIDE

A whole series of substances, some of them strongly dehydrating, upon being heated with oestrone will produce an eosin red colour. This happens when oestrone is heated together with $AlCl_3$, P_2O_5, $SnCl_2$, $ZnCl_2$ as well as in a melt of boric acid, oxalic acid or sulphosalizylic acid. Oestradiol and equilin behave in the same way. The melts of benzoic, succinic, cinnamic, salizylic and phthalic acid give negative results; $CaCl_2$ is without effect. The colours are delicate and can not be satisfactory extracted. Contrary to this, $SbCl_3$ gives strong colours and the coloured substances dissolve easily in nitrobenzene. The solutions are deep red with green fluorescence. They can be discoloured by $CHCl_3$, glacial acetic acid and especially by alcohols. This process is accelerated by heating. Methanol, ethanol, buthylalcohol, amylalcohol (furfurolfree) and octylalcohol were examined. The red nitrobenzene solution gives the red dye to Al_2O_3 in the chromatographic column; but the red ring fades quickly. — The dark colours formed between $SbCl_3$ and the accompanying impurities of urine extracts (both human and equine urines were used) are also discoloured by alcohols. These dark colours can be adsorbed by Al_2O_3 but their chromatographical separation from the red dye in the nitrobenzene solution was only partly successfull.

Method

After evaporation of the solvent there is added 1 ml 23 % solution of $SbCl_3$ in $CHCl_3$ and the tube heated for 2 min to 100°; the substance then forms a melt with $SbCl_3$; it is heated for 5 min to 160° in a glycerin-bath. 2 ml nitrobenzene are added, the tube is chilled to room temperature and the extinction read through filter S 53 or L II. The compensation-cell must be filled with nitrobenzene. The reading through L II is a little easier than through S 53 where absolute equality of the colour is difficult to achieve. Heating for more than 5 min at 160° is unnecessary; after 15 min the same values are obtained as after 5 min. When *oestradiol* (25 μg, 50 μg) is heated *10 min* at 160° the nitrobenzene solution shows a strong turbidity and the reading in the »stufo» becomes impossible. If oestrone or equilin are heated to 140° the time must be extended to 15 min. Heating to 100° does not give reproducible values. Phenols must be removed before determination as they also react with $SbCl_3$. The reaction is typical for oestrone, oestradiol and equilin, which all give a deep red coloured lake, with strong green fluorescence.

The simultaneous determination of oestrone, oestradiol and equilin gives simple additive values.

The reaction can also serve as a qualitative test to show the presence of androsterone or testosterone.

The other examined substances gave either no colour or only a very faint one and therefore could not be determined with $SbCl_3$ colorimetrically.

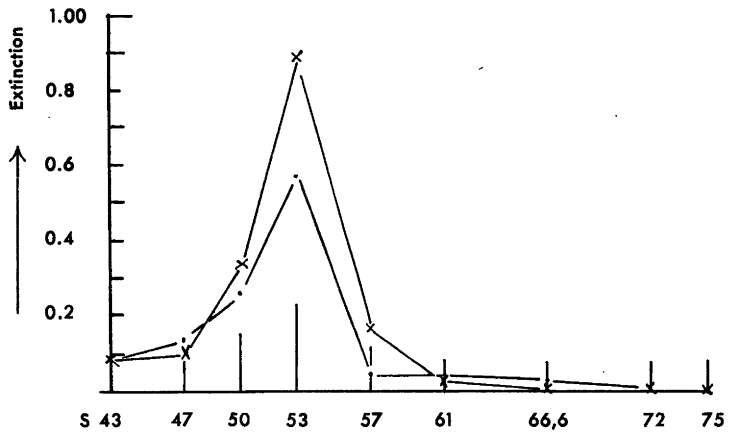

Fig. 1. Extinction on determination of 50 µg oestrone with $SbCl_3$ and $SnCl_4$. Volume 2 ml. Reading in 2.5 mm cell.

× ——— × *with* $SbCl_3$
· ——— · *with* $SnCl_4$.

The results of the experiments will be found in Tables 13—17 and curve 1.

In connection with the reaction with $SbCl_3$ will be mentioned a reaction with $SnCl_4$ communicated by Israel Kleiner [11]. As no scientific periodicals from the allied nations were available in Norway during the German occupation, the author did not know this publication. The author's investigations on the reaction with $SbCl_3$ were finished in autumn 1944.

On heating oestrone with phthalic anhydrid and $SnCl_4$ to 116°—120° a deep red substance is formed that is soluble in $CHCl_3$ with bright eosin-like colour that gradually fades away. The dye is also soluble in nitrobenzene. The author has made some experiments with the reaction of Kleiner after having read his publication after the end of the German occupation. The extinction of the nitrobenzene solution in the different wave ranges closely resembles the extinction of the coloured substance formed with $SbCl_3$ in nitrobenzene solution. The colour can also be got with $SnCl_4$ and oestrone without phthalic anhydrid; it is however less intense. The dye is very sensitive to alcohols and water. — It has not been possible to work out the reaction for quantitative purposes. Kleiner considers this dye as a phthalid; this is scarcely right as the reaction can take place without phthalic anhydrid. The great similiarity of the two extinction curves point to one and the same condensation of the substance in question under the influence of $SbCl_3$ or $SnCl_4$. The phthalic anhydrid serves only to prevent the $SnCl_4$ from escaping during the reaction, as the reaction temperature is considerably higher than the b. p. of $SnCl_4$,

Table 13. Colours on reaction with $SbCl_3$ and 50 µg substance, 5 min. at 160°.
a. Colour of the lake. b. Colour of the nitrobenzenesolution.

Substance	a.	b.
Oestrone:	purple	strong eosin-red, with strong green fluoresc.
Oestradiol:	»	» » » » » » »
Equilin:	»	» » » » » » »
Progresterone:	light brown	light yellow to orange
Testosterone:	in the beginning: deep lilac. Red dust*. Bluish at the end	faintly yellow
Androsterone:	in the beginning: yellow-brown. No dust	yellow
Pregnandiol:	faintly yellow	faintly yellow
Corticosterone:	in the beginning deep blue, light blue at the end	colourless-faintly yellow
Cholesterol:	yellow-brown	faintly yellow

Table 14. Extinction on determination of 50 µg substance with $SbCl_3$. Final vol. 2 ml. Reading in 2.5 mm cell.

	Filters									
Substance	S 43	S 47	S 50	S 53	S 57	S 61	S 66.6	S 72	S 75	L II
Oestrone	0.09	0.10	0.33	0.88	0.16	0.02	0.00	0.00	0.00	0.82
Oestradiol	0.17	0.15	0.28	0.54	0.00	0.00	0.00	0.00	0.00	0.50
Equilin	0.15	0.21	0.38	0.82	0.15	0.07	0.03	0.03	0.03	0.80
Progesterone	0.05	0.06	0.07	0.03	0.01	0.00	0.00	0.00	0.00	0.00
Testosterone	0.14	0.19	0.18	0.12	0.12	0.12	0.05	0.01	0.02	0.12
Androsterone ..	0.19	0.10	0.08	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Pregnandiol**....	0.07	0.06	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.05
Corticosterone ..	0.16	0.17	0.22	0.16	0.16	0.23	0.04	0.03	0.00	0.16
Cholesterol	0.09	0.09	0.11	0.06	0.06	0.02	0.02	0.02	0.00	0.02

Table 15. Validity of Beer's law on reaction with $SbCl_3$. Final vol. 2 ml. 2.5 mm cell.

		Quantities in ug			
Substance	filter	25	50	100	
Oestrone	S 53	0.45	0.88	1.78	(reduced from 1 mm)
Oestradiol	S 53	0.28	0.54	1.08	
Equilin	S 53	0.42	0.82	1.65	(reduced from 1 mm)

Contrary to oestrone, oestradiol and equilin all other examined substances show bad agreement with Beer's law for nearly all examined filter ranges.

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* »Red dust», look at Tables 9 and 10.

** 100 µg pregnandiol gives the same values!

Table 16. Simultaneous determination of oestrone, oestradiol and equilin with $SbCl_3$. Final vol. 2 ml. Reading with 2.5 mm cell. 25 µg each substance.

	Filters								
Substance	S 43	S 47	S 50	S 53	S 57	S 61	S 66.6	S 75	L II
Oestrone + oestradiol	0.09	0.14	0.32	0.78	0.19	0.07	0.02	0.04	0.77
Calculated:	0.10	0.12	0.31	0.75	0.19	0.08	0.03	0.03	0.76
Oestrone + equilin	0.14	0.20	0.39	0.84	0.18	0.07	0.04	0.02	0.82
Calculated:	0.14	0.20	0.39	0.87	0.15	0.07	0.04	0.01	0.86
Oestradiol + equilin	0.15	0.16	0.31	0.70	0.16	0.06	0.04	0.00	0.68
Calculated:	0.16	0.22	0.36	0.72	0.16	0.09	0.05	0.01	0.72
Oestrone + oestradiol + equilin	0.22	0.28	0.51	1.12	0.24	0.08	0.05	0.03	1.04
Calculated:	0.20	0.27	0.53	1.17	0.26	0.12	0.06	0.01	1.18

Table 17. Extinction of the lake produced with 100 µg oestrone and $SnCl_4$ (reaction of Israel Kleiner). The lake is dissolved in 5 ml $CHCl_3$. Reading with 5 mm cell.

	Filters									
Substance	S 43	S 47	S 50	S 53	S 57	S 61	S 66.6	S 72	S 75	L II
Oestrone	0.16	0.26	0.52	1.14	0.08	0.08	0.06	0.00	0.00	0.98

but under that of the m. p. of phthalic anhydrid. Moreover the result of the reaction is uncertain unless care is taken to get a homogenous melt through a short heating above the m. p. of the phthalic anhydrid, before dropping in the $SnCl_4$. The results will be found in Table 17 and Fig. 1.

THE REACTION WITH THE REAGENT OF FOLIN-WU

The reagent of Folin-Wu (phospho-molybdo-tungstic acid) can be used for the determination of absolutely pure oestrone. A blue colour is developed as the result of a reducing process.

Method

To the substance is added 0.5 ml conc. H_2SO_4 and the tube is heated for 2 min to 100°, cooled and 2 ml of the reagent of Folin-Wu are dropped in. The tube is now heated for 5 min to 100°, cooled and 5 ml saturated solution of sodium carbonate added. Then the tube is heated again to 100° for 3 min, cooled and read in the »stufo» through filter

S 72. The success of the reaction depends on working in an acid medium also after the addition of the carbonate solution. If not, the development of the blue colour will come to an early standstill and Beer's law is not valid. In a separate experiment the amount of saturated carbonate solution which is necessary must be determined for the exact neutralisation of the Folin-Wu solution. For the real experiment a little less must be taken. For the author's work 5 ml saturated carbonate solution were used while 5.8 ml were needed for the neutralisation.

The results are given in Table 18. Since even traces of impurities may cause trouble, only very pure samples of oestrone can be determined in this way.

Table 18. Validity of Beer's law on determination og oestrone with the reagent of Folin-Wu. Final vol. 7.5 ml. Reading with 10 mm cell.

		Quantities in μg oestrone		
Substance	Filter	250	500	1000
Oestrone	S 66.6	0.64	1.28	2.60
Oestrone	S 72	0.77	1.49	3.00*

DISCUSSION

Above some colour reactions have been described that can be used for quantitative determination of certain substances with hormonal properties from the steroid group. They are distinguished by a considerable sensitivity. They are typical for the examined substances in so far as the presence of troublesome impurities can be excluded either as a result of previous purification or from the nature of the substance in question. All the reactions described are sensitive to impurities. On filtering solutions containing lipoid solvents through qualitative filterpaper (Norwegian war-time manufacture) substances (resins) were dissolved that made the described reactions impossible in the filtrate. Owing to this glass filter crucibles were used for all filtrations. As conc. H_2SO_4 reacts even with traces of impurities experiments were carried out to find a substitute for the conc. H_2SO_4. Both phosphoric acid (85 %) and pyrophosphoric acid were tried, but without any success.

Experiments carried out in connection with this work — to purify extracts from mare's urines and from human urines, so far as their content of oestrogenic substance could be determined with one of the described reactions — were without success. The extensive purification of such extracts with the reagent »Girard P» could not be tried as the reagent was unobtainable during the occupation.

* Reduced from reading in 5 mm cell (found: 1.50).

SUMMARY

The author communicates some reactions that can be used for the quantitative determination of oestrone, oestradiol, equilin, androsterone, testosterone, corticosterone and cholesterol.

1. Reaction with *p*-dimethylaminobenzaldehyde (reagent P) that will allow the determination of 0.5 μg using 10 mm microcells.

2. Analogous reaction with *m*-nitrobenzaldehyde (reagent M) and *p*-nitrobenzaldehyde.

3. Reaction with salizylaldehyde (reagent S) that permits the seizure of the said substances qualitatively and quantitatively.

4. Reaction with $SbCl_3$ by the aid of which oestrone, oestradiol and equilin can be determined quantitatively, while the other examined substances do not give the deep red colour that is typical for the said 3 substances.

5. The working conditions are given for the determination of oestrone with the reagent of Folin-Wu.

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The Distribution of Sizes of Particles in Some Polymeric Methyl Methacrylates *

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By the sedimentation of polydisperse substances in the ultracentrifuge a concentration gradient will appear in the cell due to the various sedimentation velocities of particles with different molecular weights. The shape of the concentration gradient curve will depend upon the nature of the frequency function of the sedimentation constants or — as the sedimentation constant is some function of the molecular weight — the frequency function of the molecular weights. From a sedimentation diagram, therefore, it must be possible to deduce the distribution of sizes of particles in a polydisperse substance.

Since the development of the ultracentrifugal technique this method has been used by Rinde [1] to obtain the frequency curves for the particles in gold sols, by Nichols *et. al.*[2] on suspensions of ferric oxides, barium sulphates, on emulsions and rubber latex etc. More recently Jullander [3] has thoroughly discussed different ways of obtaining frequency curves for cellulose nitrates. In this connection the work of Signer and Gross [4] on polystyrene can also be mentioned. The earlier work has mainly been concerned with substances, the frequency curves of which show only one maximum. Further their sedimentation has been almost independent upon the concentration; an exception is of course the behaviour of cellulose nitrate and polystyrene.

When the frequency curve of the substance under investigation has more than one maximum and when the sedimentation velocity is dependent upon the concentration, the problem of obtaining the frequency curve from the sedimentation diagram is very complicated. In this paper some of the prob-

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