Ester cholesterol. Fluid B, supernatant after the free cholesterol has been separated as digitonide, contains cholesterol esters, neutral fats and excess digitonin. After the excess digitonin has been precipitated as indicated for the determination of total fatty acids (treatment of fluid A), the supernatant fluid is decanted, evaporated and saponified, extracted with L.P., and the cholesterol determined by precipitation with digitonin as described for total cholesterol. If desired, the fatty acids derived from neutral fats and cholesterol esters can also be separated in the same way as total fatty acids.

Table 1 shows the amounts of cholesterol recovered after it has been added, dissolved in CHCl_3 , to ethanol-ether extracts of known cholesterol content. The accuracy of the method is further illustrated by the results given in Table 2, which are examples of determinations of total, free and ester cholesterol on various tissue extracts.

Table 1. *Recovery of cholesterol added in CHCl_3 solution to ethanol-ether extracts of tissues*

Cholesterol added (mg.)	Cholesterol recovered (mg.)	Cholesterol added (mg.)	Cholesterol recovered (mg.)
0.200	0.199	0.500	0.496
0.200	0.199	0.500	0.502
0.200	0.198	0.500	0.498
0.200	0.200	0.500	0.500
0.400	0.400	1.000	1.000
0.400	0.400	1.000	1.000

Table 2. *Results of total, free and ester cholesterol determinations in extracts of various tissues*

Material	Cholesterol content mg./100 g. moist tissue		
	Total	Free	Ester
Human kidney cortex	220	202	22
	224	198	27
	381	358	23
	326	219	107
Human kidney medulla	193	137	45
	230	171	60
	255	194	64
Human liver	252	225	30
Human adrenal	2500	420	2100
Human plasma	152*	65*	85*
Human heart	88	74	12

* mg./100 ml. of plasma.

SUMMARY

A method is described for determination of total, free and ester cholesterol in tissue extracts by a combination of the method of Schoenheimer and Sperry (modified by Sturges and Knudson) with that of Kelsey.

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Adrenaline and Related Substances in Blood and Tissues

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The results obtained with the colorimetric method of Shaw [1938], for the assay of adrenaline in blood and tissues, cannot be attributed to adrenaline alone, since the denominator of the 'specific ratio', i.e. the ratio of the results with acid and with alkali [cf. Shaw, 1938], was found, with a few exceptions, to be considerably lower than that of adrenaline itself (Table 1). Quantitative adsorption of ascorbic acid by $\text{Al}(\text{OH})_3$ at a pH of about 8.5 has been regularly observed by the writer, and thus ascorbic acid must be regarded as participating in the colorimetric results, contrary to a statement by Shaw [1938]. Despite the low value of the denominator of the specific ratio (henceforth designated d.s.r.) for ascorbic acid (0.3), this substance alone cannot

be responsible for the low d.s.r.'s generally found in blood and most tissues, including the adrenal glands (Table 1). Its chromogenic intensity is too weak (Table 2) and its reported average concentrations in blood and tissues (Table 3) are too small.

Many chromogenic substances other than adrenaline and ascorbic acid are eliminated by the adsorption procedures of the method. Certain chromogenic hormonal steroids, which are by themselves not adsorbed on $\text{Al}(\text{OH})_3$, were originally believed [Raab, 1941 a, b, c] to be partially adsorbed at pH 8.5 in the presence of adrenaline, owing to the formation of a hypothetical 'adreno-cortical' compound. This conception is now abandoned since it was found that steroids pass quantitatively into

Table 1. *Normal averages of colorimetric results obtained from tissues and blood*

Organ	Content of adsorbable chromogen (colour units/g. or ml.)	Denominator of the specific ratio*	No. of specimens
Adrenal glands			
(rat)	259,311	1.26	56
(man, medulla)	232,000	1.33	6
(man, cortex)	23,820	1.51	6
Spleen (rat)	2,322	1.00	20
Testis (rat)	1,960	1.07	2
Brain (rat)	1,775	0.89	19
Liver (rat)	1,765	0.98	26
Kidney (rat)	1,180	1.08	28
(man)	1,438	1.32	14
Heart (rat)	946	1.00	40
(man)	698	1.37	21
Muscle (rat)	898	1.88	19
Aorta (man)	493	3.50	17
Renal artery (man)	258	3.55	8
Blood (man)	156	1.06	51
(dog)	168	?	3
(cat)	123	?	2

* A value greater than 2.0 indicates pure or almost pure adrenaline, or sympathin; a d.s.r. between 2.0 and 1.0 indicates the presence of both adrenaline and other adsorbable chromogens with a d.s.r. lower than that of adrenaline; a d.s.r. of 1.0 or below indicates the exclusive presence of adsorbable chromogens other than adrenaline or sympathin.

Table 2. *Chromogenic properties and adsorbability of some substances related to adrenaline*

Substance	Colour intensity compared with that of adrenaline	Denominator of the specific ratio	Adsorbability at pH 8.5
Adrenaline	1	2.0-3.5*	+
Ascorbic acid	1/320	0.3	+
Adrenalone	1/11	1.1	+
Dihydroxyphenylalanine	1/3	1.1	+
Epinephrine (synthetic)	1/6	1.1	+
Leuko-adrenochrome (?)	?	1.0	+
Pyrocatechol	1/9	0.6	+
Adrenochrome	1/5	1.0	-
Benzoquinone	1/10	2.4	-
Quinol	1/20	2.7	-

* These d.s.r.'s are found if adrenaline is subjected to the adsorption procedures; without these it is about 5.0 [cf. Shaw, 1938].

Table 3

The average amounts of ascorbic acid present in various tissues according to data collected from the literature would have to be multiplied by the figures given below in order to lower the d.s.r. of pure adrenaline to the d.s.r.'s actually found in the following tissues:

Adrenal glands	10-20	Heart muscle	7
Spleen	2	Striated muscle	1-1½
Liver	2	Brain	3-4
Kidney	3-4	Blood	4-5

organic solvents (chloroform, petroleum ether) from aqueous solutions containing adrenaline, while the latter remains in the water. The original erroneous conception had been based on the following facts: (a) increased colour effect and lowered d.s.r. of adrenaline solutions to which cortical and other steroids had been added; (b) quantitative recovery of the entire chromogenic material in chloroform extracts of dried blood and tissues. The first phenomenon is probably to be explained by an alteration of the adrenaline molecule, rather than by a combination with the added steroids; the second one seems to be due to the fact pointed out by Kendall [1942] and confirmed by the writer, that adrenaline, which is otherwise chloroform-insoluble, becomes chloroform-soluble in the presence of lecithin. The same was found to be true also for ascorbic acid and adrenalone.

If a chromogenic substance is to be estimated by Shaw's method, it must be adsorbed by $\text{Al}(\text{OH})_3$ at a pH of about 8.5. A search for adsorbable substances which might account for the low d.s.r.'s found in blood and tissues revealed the fact that this condition was fulfilled only by such chromogenic substances as possessed two or more adjacent OH groups on a benzene ring or heterocyclic ring containing oxygen. Thus, besides adrenaline proper and ascorbic acid, a number of adrenaline-like substances with an intact catechol nucleus were found to be detectable with the method used (Table 2). Pyrogallol and gallic acid which, by virtue of their adjacent hydroxyl groups, are also adsorbable, are probably not present in blood and tissues.

The integrity of the catechol nucleus of adrenaline and related substances appears to be of fundamental significance for the adsorbability by $\text{Al}(\text{OH})_3$ in an alkaline medium, as well as for the especially high electrical potential of adrenaline and related catechols [Ball & Chen, 1933] and, according to Tainter [1930] and Vaughan, Perkins & Derbes [1942], for the sympathomimetic properties of these substances. Adrenochrome and quinones give a somewhat intense colour reaction (Table 2), but, lacking OH groups in adjacent positions, they are not adsorbable by $\text{Al}(\text{OH})_3$ at pH 8.5. Their electrical potential is lower than that of catechol compounds [Ball & Chen, 1933] and their pharmacodynamic effects are of a prevalently depressant character, such as is the case with adrenoxin [Heirman & Bacq, 1940], and other quinones [Oster & Sobotka, 1942]. The conclusion is reached that, except in those instances in which a d.s.r. greater than 2.0 indicates the presence of pure or almost pure adrenaline [or sympathin; Cannon & Lissák, 1939], the results obtained with Shaw's method are due not only to adrenaline itself and to ascorbic acid, but also to certain adrenaline-like catechol compounds with a d.s.r. lower than that of adrenaline.

Even the isolated human adrenal medulla did not yield pure adrenaline. Its very high content of adsorbable chromogenic material (Table 1) had an average d.s.r. as low as 1.33. The d.s.r.'s of rat adrenals were similarly low and showed only insignificant alterations under various experimental conditions.

The colorimetric results of Shaw's method as applied by Raab [1941 *a, b*] are expressed in 'colour units', each unit corresponding to the chromogenic intensity of 0.001 $\mu\text{g.}$ of adrenaline.

The adrenal glands contain by far the largest amounts of adsorbable chromogen (Table 1). Its concentration in the cortex of human glands was about one-tenth of that in the medulla, possibly due in part to post-mortem contamination.

Relatively large amounts of an adsorbable chromogen not identical with adrenaline itself were also present in liver, kidney and brain (Table 1), organs which are known to remove adrenaline most efficiently from perfusing fluids [Bernheim, 1942] and to contain the highest concentrations of an amine-oxidase which destroys adrenaline through oxidative deamination of its side-chain [Blaschko, Richter & Schlossmann, 1937]. The striated muscle, on the other hand, in which the adrenaline-destroying oxidase is not present [Blaschko *et al.* 1937] contained mainly adrenaline, and the proportion was even higher in the tissue of arterial walls. Since muscular tissue has only a weak tendency to remove adrenaline from perfusing fluids [Pak, 1926], it seems probable that at least part of its content in chromogenic material with a high d.s.r. is identical with sympathin deriving from peripheral adrenergic neurones [Cannon & Lissák, 1939].

The lowest content of adsorbable chromogen was found in blood with a d.s.r. close to 1.0 under standard conditions, which indicates that adrenaline itself, if it is present in blood, circulates in very small amounts only. Bloor & Bullen [1941], using another modification of Shaw's method, came to the same conclusion.

The doubtful specificity of Shaw's method made it desirable to investigate its usefulness as a criterion of adrenal activity by correlating its results with a variety of experimental and clinical conditions which are known or suspected to be accompanied by alterations of adrenal secretion or hormone deposition.

More than 3000 determinations were carried out for this purpose with the following results, some of which have been published in detail [Raab, 1941 *a, b, c, d, e*, 1942]. The term 'chromogen' signifies material which is adsorbed by $\text{Al}(\text{OH})_3$ at a pH of about 8.5 and estimable by Shaw's method.

(1) *Injection of adrenaline* (0.5–1.0 mg.) was followed within 30 min. by an average increase of the d.s.r. of human blood from 1.11 to 1.58, while the total level of chromogen showed only an insignificant elevation (10 exp.). In rats,

both the concentration of chromogen and the d.s.r. were increased in the kidney, liver, brain and particularly in the heart muscle (+138%). The latter displayed an outstanding tendency to accumulate both injected adrenaline and other catechol compounds (adrenalone, dihydroxyphenylalanine, pyrocatechol) (52 exp.).

(2) *Injection of thyroxin* in rats increased the myocardial d.s.r. (10 exp.). After pretreatment with thyroxin the myocardial accumulation of injected adrenaline was intensely accelerated and increased with resulting cardiac death within a few minutes (6 exp.).

(3) *Injection of desoxycorticosterone acetate* in man was followed by a slight increase of the d.s.r. of the blood (12 exp.). In rats it was followed by an increased chromogen content of kidney, liver and heart muscle with only little change of the d.s.r. (30 exp.). The vasopressor effect of adrenaline was markedly increased by pretreatment with desoxycorticosterone acetate (6 exp.).

(4) *Injection of insulin*. 10–15 units applied intravenously increased the d.s.r. of human blood from 1.09 to 1.40 within 45 min. (5 exp.). In rats the chromogen concentration and d.s.r. of the heart muscle, liver, brain and striated muscle were increased (40 exp.).

(5) *Vitamin B₁ deficiency*. The myocardial adsorbable chromogen was markedly increased in rats. Administration of thiamin chloride restored conditions to normal within a few days (15 exp.).

(6) *Exposure to cold*. Increase of chromogen and d.s.r. in the heart muscle of rats; in the striated muscle the total chromogen was diminished but the d.s.r. was increased (20 exp.).

(7) *Physical exercise*. In fourteen normal persons a slight elevation of the average blood chromogen and an elevation of the d.s.r. from 0.86 to 1.28 took place within 2–5 min.; return to normal followed within 15 min. In rats a considerable increase of the myocardial chromogen and d.s.r. occurred (30 exp.) and in the striated muscle a diminution of the total chromogen accompanied by an increase of the d.s.r. (30 exp.).

(8) *Adrenalectomy* in rats was followed by a diminution of the chromogen of the heart muscle (22%), kidney (7%), liver (33%), brain (24%), striated muscle (19%) and spleen (47%), with only little change of the d.s.r. (60 exp.). In one adrenalectomized dog the blood chromogen level decreased steadily without, however, falling below the lowest limit of normal.

(9) *Essential hypertension*. At rest a normal blood chromogen level in 36 cases, but an increased average elevation of the blood chromogen after exercise (14 exp.).

(10) *Angina pectoris*. In twelve cases a normal chromogen level at rest, but abnormally high elevations of the blood chromogen after exercise (average from 140 to 195 colour units/ml.).

(11) *X-ray irradiation of the adrenal region* diminished the myocardial chromogen of rats after exercise (14 exp.). In seven patients with angina pectoris the abnormal chromogen discharges were abolished together with the anginal symptoms for periods of several months.

(12) *Pulmonary oedema* in two cardiac patients was accompanied by an abnormal increase of the blood chromogen level.

(13) *Kidney insufficiency* with uræmia was regularly accompanied by a strikingly elevated chromogen level of the blood (average 372 colour units/ml.) with an average d.s.r. of 1.13, probably due to impaired excretion (17 cases).

(14) *Cushing's disease*. Four cases showed a blood chromogen level near the upper limit of the normal range (average 215 colour units/ml.).

(15) *Addison's disease*. In one mild case the chromogen level was moderately below the normal average (98 colour units/ml.).

(16) *'Hypertensive' and 'arteriosclerotic' heart disease with cardiac failure*. In the heart muscle either the total chromogen or the concentration of adrenaline proper was abnormally high in 78% of 32 cases. In one case the myocardial chromogen was abnormally low after therapeutic sympathectomy, which may be comparable with the disappearance of chromogenic material from the cat's heart after denervation, as observed by Cannon & Lissák [1939].

(17) *Unexplained sudden cardiac death* of a young athlete. The chromogen concentration of the heart muscle was excessively high (1622 colour units/g. against an average of 698 in 21 normal hearts).

(18) *Ageing and arteriosclerosis*. The average chromogen concentrations of heart, kidneys and arterial walls were found to be lowest in early childhood. They increased gradually and reached a maximum in senile age. Particularly high chromogen concentrations were found in arteriosclerosis (138 specimens).

(19) *Adrenal tumours*. In two cases with adrenal tumours, abnormally high chromogen concentrations were found in arterial walls and kidneys.

SUMMARY

1. The results obtained with the method of Shaw [1938] for the assay of 'adrenaline' in blood and tissues (adrenal glands, liver, spleen, kidney, brain, heart) are due to the presence in these tissues not only of adrenaline itself, but also of adrenaline-like substances which contain a catechol nucleus, and of ascorbic acid. Thus, although the method is not specific for adrenaline, it can be used for investigations of adrenal and adrenergic function in a broader sense.

2. The results of numerous determinations carried out under various experimental and clinical conditions are in general accord with existing knowledge or concepts regarding adrenal and adrenergic physiology and pathology.

3. Contrary to the author's earlier view, it is no longer held that the low denominator of the 'specific ratio' obtained by Shaw's method for many tissues is due to the presence of adrenaline in combination with cortical hormones. This ratio is low when adrenaline-like compounds or ascorbic acid are present.

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Studies on Cholinesterase

3. SPECIFIC TESTS FOR TRUE CHOLINESTERASE AND PSEUDO-CHOLINESTERASE

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In the animal body two esterases capable of hydrolysing acetylcholine exist: a true cholinesterase, acting exclusively on certain choline esters, and a non-specific enzyme, which also hydrolyses a variety of non-choline esters [Mendel & Rudney, 1943]. The non-specific or pseudo-cholinesterase, purified from horse serum and from dog pancreas [Mendel & Mundell, 1943], exhibits its maximum activity at

high concentrations of acetylcholine (above 300 mg./100 ml.), and is far less active at low substrate concentrations; the specific or true cholinesterase, on the other hand, found in red blood cells and brain tissue, displays its greatest activity at low substrate concentrations (below 3 mg./100 ml.) and is strongly inhibited at high concentrations of acetylcholine.