Renal Clearance of 17-Oxo Steroid Conjugates Found in Human Peripheral Plasma

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The widespread practice of using urinary 17-oxo steroid determinations as a means of studying disorders of androgen metabolism assumes the existence of some relationship between the amount of androgens secreted in the body and the amount of 17-oxo steroid excreted in the urine. There is good evidence that the 17-oxo steroids arise from biologically active steroids formed in the adrenals and testes, although it is uncertain where these compounds are metabolized to the conjugated 17-oxo steroids found in peripheral plasma and urine. Whether or not urinary excretion patterns of steroids accurately reflect the concentration of steroids in the plasma depends to a large extent on the renal clearance of androgen metabolites in circulation in blood. It is clearly desirable to determine simultaneously blood and urine levels for 17-oxo steroids and to establish renal clearance values for these compounds.

The existence of free 17-oxo steroids in urine is very doubtful. Reliable methods of determination give very low figures, and the possibility of hydrolysis after collection cannot be excluded. By contrast, most 17-oxo steroids present in urine are liberated by procedures which hydrolyse glucuronide and sulphate conjugates.

Bush (1956) reported the presence of dehydroepiandrosterone (3β -hydroxyandrost-5-en-17-one), androsterone and 11β -hydroxyandrost-4-ene-3:17-dione in adrenal-vein blood from a woman with post-pubertal virilism. Romanoff, Hudson & Pincus (1953) found androst-4-ene-3:17-dione and its 11β -hydroxy derivative in two samples of adrenal-vein blood. These unconjugated 17-oxo steroids have not been found in peripheral blood. Migeon & Plager (1954) and Migeon (1956) showed that dehydroepiandrosterone and androsterone are present in peripheral blood as sulphates which are hydrolysed by continuous extraction with ether at pH 0.8. According to Migeon, little if any steroid could be extracted from either non-hydrolysed plasma or plasma hydrolysed with β -glucuronidase. Clayton, Bongiovanni & Papadatos (1955) stated that they found 171 μ g. of 17-oxo steroid/100 ml. in a large sample of blood, and that of this 12% was present as glucuronide. The ratio of the concentration of 17-

oxo steroids in urine to that in plasma was 4.1 for glucuronides and 0.57 for sulphates.

As it is known that in normal urine samples the 17-oxo steroids conjugated as glucuronides form more than half the total, the failure of Migeon to find 17-oxo steroid glucuronides in plasma suggests a high clearance rate for this form of conjugate. This is also apparent from the results of Clayton *et al.* This paper describes a method for the determination of 17-oxo steroid glucuronides and sulphate levels in plasma and urine. The clearances calculated from results obtained from normal subjects showed that, whereas the glucuronide conjugates are probably excreted mainly by glomerular filtration, the sulphate-bound 17-oxo steroids may be either excreted by glomerular filtration and partially re-absorbed, or else excreted by the renal tubules.

MATERIALS

β -Glucuronidase. This was a preparation from limpets (*Patella vulgata*) (powder B of Dodgson & Spencer, 1953) with activity of 700 000 units/g., *p*-chlorophenol glucuronide being used as substrate (Spencer & Williams, 1951).

Other materials. Alumina (Savory and Moore): 120–200 mesh, containing 4.4% of water; *m*-dinitrobenzene: 1% (w/v) (twice recrystallized from ethanol) in ethanol; 2.5N-KOH: in ethanol containing 30 mg. of ascorbic acid/100 ml. (Wilson, 1954); sodium acetate buffer: 0.5M, pH 4.0; ether and carbon tetrachloride: distilled; benzene: distilled and water-free; ethanol: re-rectified.

METHOD

Blood. Blood samples (50 ml.) were drawn from an arm vein into heparin (1000 units) and were centrifuged at 1550 g for 15 min. All the samples studied were obtained between 10 and 12 a.m. The plasma was removed and stirred vigorously while the protein was precipitated by the slow addition of 4 vol. of ethanol. The precipitate was removed by centrifuging (980 g for 5 min.) and was washed with 1 vol. of ethanol-water (4:1, v/v). The combined aqueous ethanol solutions were evaporated almost to dryness at 50° under reduced pressure (1 cm. mercury) and the residue was transferred to a stoppered centrifuge tube with water (25 ml.) and benzene (25 ml.). This mixture was shaken for 2 min., and after centrifuging (980 g for 10 min.) the clear yellow benzene layer was removed. To ensure the removal of fat the aqueous phase was extracted three or more times with benzene until the final extract was colourless.

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Hydrolysis of 17-oxo steroid glucuronides

The remaining aqueous layer was evaporated under reduced pressure almost to dryness and the residue was transferred to a stoppered tube with 0.5M-acetate buffer (20 ml.; pH 4.0). Potassium dihydrogen phosphate (100 mg.) was dissolved in the buffer solution (to inhibit the action of the sulphatases in the enzyme preparation) and 50 mg. of β -glucuronidase homogenized in buffer (5 ml.) was added. As a bacteriostatic agent penicillin (50 000 units) was added and the mixture incubated at 47° for 16 hr. Material liberated by the enzyme preparation was extracted with benzene (3 $\times$ 10 ml.) and centrifuged to break emulsions formed. The combined benzene extracts were evaporated to approx. 1 ml., and carbon tetrachloride (20 ml.) was added. This solution was washed with 2N-NaOH (3 $\times$ 2 ml.) and with water until neutral, and finally evaporated to dryness. The material thus obtained by β -glucuronidase hydrolysis was unsuitable for direct chromatographic separation, as it contained some material which impeded the flow of developing solvents through the narrow alumina column. The residue was therefore dissolved in benzene (5 ml.) and transferred to a wide column (1 cm. diam.) of alumina (2 g.). A preliminary wash of this column with benzene (15 ml.) contained no Zimmermann-positive material and the 17-oxo

steroids were then eluted with a 2% (v/v) solution of ethanol in benzene (20 ml.). The ethanolic benzene solution was evaporated to dryness to give the residue containing 17-oxo steroids for chromatographic separation.

Hydrolysis of 17-oxo steroid sulphates

The aqueous enzyme digest which had been extracted with benzene was acidified with 2N-HCl (10 ml.) and extracted continuously with ether in a liquid-liquid extractor (150 ml.) for 48 hr. The pH after extraction was approx. 0.6. The ether solution (100 ml.), after being washed with 2N-NaOH (3 $\times$ 10 ml.) and with water until neutral, was finally evaporated to dryness to give the residue containing 17-oxo steroids for chromatographic separation.

Urine. Each subject drank water (300 ml.) and the bladder was emptied 1 hr. before the blood sample was obtained. The urine passed during the following 2 hr. period was collected, and the conjugated steroids were extracted by the method of Edwards, Kellie & Wade (1953) and worked up in the same way as the plasma extract. The 17-oxo steroid content of the glucuronide and sulphate fractions were determined on portions of the extracts by the modification of the Zimmermann reaction described by Callow, Callow & Emmens (1938).

Fig. 1. Reservoir apparatus for supplying solvent gradient to the alumina column under pressure. Pressure is applied at A, and the solvent outlet at B is connected to the column with 1 mm. polythene tubing. As solvent leaves the stirred tube, more polar solvent enters through the siphon. The stirrer is actuated magnetically from an electric motor.

Fig. 2. Bottle of 2 l. capacity fitted for applying pressures up to 15 cm. mercury. With the stopcock closed the pressure may be raised by means of the rubber bulb and the connecting rubber tube then clamped.

Final chromatography of the 17-oxo steroid fractions

The residues obtained from blood and from portions of the final extracts from urine containing approx. 50 $\mu\text{g.}$ of total 17-oxo steroids were chromatographed on a column of alumina (1.5 g.; height 16 cm. $\times$ 0.325 cm. internal diam.). The material was transferred to the column in benzene (3 $\times$ 0.1 ml.) and the column was developed by gradient elution (Kellie & Wade, 1957) donating 2% (v/v) ethanol in benzene (35 ml.) into a recipient vessel containing benzene (50 ml.).

It was necessary to apply pressure (approx. 4 cm. mercury) to obtain a flow rate of 10 fractions (0.5 ml.)/hr., and the apparatus illustrated in Fig. 1 was devised for this purpose. The twin vessels of the gradient-elution reservoirs were made from two Pyrex standard ground-glass joints (B19 and B34 for donor and recipient respectively). The two sockets were sealed at the unground ends and were joined by a tube (5 mm. internal diam.) near the standard joints and by a siphon capillary tube (1 mm. internal diam.) at the sealed end. A capillary outlet from the B34 recipient vessel was connected to the column by a short length of polythene tubing (1 mm. internal diam.). A magnetic stirrer, operating in the recipient vessel, was mounted by two ball races in a brass collar turned to a B34 cone. This brass collar was sealed by a polythene disk (one-eighth inch thick) held in position by a flat brass ring secured by screws. The stirrer was driven by a second magnet mounted on the spindle of an electric motor outside the polythene seal.

To set up the apparatus the benzene (50 ml.) was placed in the recipient vessel and the 2% (v/v) ethanol in benzene (33 ml.) in the donor vessel, and the stirrer placed in position and set in motion. The siphon was then filled by applying suction to the side tube of the siphon and was maintained by a screw clip on the rubber tube joined to it. When the 17-oxo

steroid material had been adsorbed on the alumina, benzene (1 ml.) was run on to the top of the column and the reservoir was immediately connected to it. Pressure (approx. 4 cm. mercury) was applied to the column through a B19 standard ground-glass joint (at A, Fig. 1) from a bottle of 2 l. capacity fitted with a 15 cm. mercury U-tube and a side arm connected to a rubber-bulb pump (50 ml.) (Fig. 2). The air in the pressure bottle was kept dry by placing a few grams of anhydrous CaCl_2 in the bottom of the bottle. This arrangement gave 10–12 fractions (0.5 ml.)/hr., and the 120 fractions were collected overnight by an automatic-fraction collector. Large stocks of the eluting solvents were prepared initially, and by using these and the same batch of alumina throughout, the positions of the eluted 17-oxo steroids could be determined by reference to a standard chromatogram. The positions of these steroids were checked by determining the 17-oxo steroid content of intermediate fractions (e.g. fractions 53 and 62 in Fig. 3), by a one-twentieth-scale modification of the Zimmermann reaction (Kellie & Wade, 1957) and reading the optical density in a micro cell (0.5 ml.; optical path 1 cm.) with a spectrum green filter (Ilford 604).

The fractions containing the individual compounds were combined and the 17-oxo steroid content of each sample was determined by a one-quarter-scale Zimmermann reaction. A volume (0.1 ml.) of 1% (w/v) *m*-dinitrobenzene and 0.05 ml. of 2.5*N*-ethanolic KOH were added to the residue, and after incubation at 25° for 1 hr. the solution was diluted with 2.5 ml. of ethanol and read in a spectrophotometer (Unicam 600; optical path 1 cm.) at 440, 520 and 600 $\mu\text{m.}$ Simultaneously a reagent blank and a standard (5 $\mu\text{g.}$ of dehydroepiandrosterone) were run and the corrected extinction was found by Allen's method (1950). The identity of the compounds so determined was checked by paper chromatography of the material recovered from the reaction solution (Kellie & Smith, 1956).

Fig. 3. Chromatograms of 17-oxo steroids. 84 $\mu\text{g.}$ from cold acid hydrolysate of urine H.B. Fractions 46–51: 3 β -hydroxy-androst-5-en-17-one (dehydroepiandrosterone); fractions 54–61: androsterone; fractions 64–71: 3 α -hydroxy-5 β -androstan-17-one (aetiocolanolone). Fractions 85–93: 10 $\mu\text{g.}$ of 3 α -hydroxy-5 β -androstan-11:17-dione; fractions 107–115: 40 $\mu\text{g.}$ of 3 α :11 β -dihydroxy-5 β -androstan-17-one.

In the material obtained from urine by dilute acid hydrolysis a red pigment occurred which when present in relatively large amounts was not completely separated from dehydroepiandrosterone by chromatography. To determine the steroid content of those fractions which contained pigment they were divided into two equal portions, one of which was used in the absence of *m*-dinitrobenzene as a pigment blank against the one-twentieth-scale Zimmermann reaction carried out on the other portion. This difficulty did not arise with blood samples or with urine glucuronide preparations; in occasional urine sulphate preparations which contained large amounts of dehydroepiandrosterone a small proportion of this was found in adjacent fractions containing pigment.

RESULTS

The concentrations of dehydroepiandrosterone sulphate and androsterone sulphate found in plasma and urine samples are given in Table 1 together with the calculated clearances. Within this small group of normal subjects the plasma levels of both dehydroepiandrosterone (4.7–131 $\mu\text{g./100 ml.}$) and androsterone sulphate (1.0–43 $\mu\text{g./100 ml.}$) show wide variation. Samples were collected from female subjects without relation to the events of the menstrual cycle. With the exception of three

instances the clearance values lie below 10. The corresponding data for androsterone and 3 α -hydroxy-5 β -androstan-17-one (aetiocholanolone) glucuronides are given in Table 2. The renal clearances are calculated from the formula 'Ratio of concentrations in urine and in plasma multiplied by the volume of urine excreted/min.' and are based on the assumption that the kidney does not play an active part in the formation of conjugates. These values are high and indicate that the glucuronides of androsterone and aetiocholanolone are cleared at about the same rate as inulin. In the larger samples of plasma collected, small amounts of 3 α -hydroxy-5 β -androstan-11:17-dione (0.3 $\mu\text{g./100 ml.}$ in sample K.W.) and 3 α :11 β -dihydroxy-5 α -androstan-17-one (0.9 $\mu\text{g./100 ml.}$ in sample J.W.) were determined and identified. No trace of dehydroepiandrosterone was found in the urine glucuronide fractions, although small amounts of this compound were identified in the glucuronide fraction of two of the three large plasma samples (1.7 $\mu\text{g./100 ml.}$ in K.W.; 2.8 $\mu\text{g./100 ml.}$ in E.K.). Roy (1956) found that limpet sulphatase in 0.005 *M*-phosphate had 2% of the optimum activity in the absence of phosphate. The sulphatase in the enzyme

Table 1. 3 β -Hydroxyandrost-5-en-17-one (dehydroepiandrosterone) and androsterone in sulphate fractions from plasma and urine

Age range of males: 20–40 years; females: 20–30 years.

	Plasma vol. (ml.)	Dehydroepiandrosterone				Androsterone		
		Urine (ml./min.)	Urine ($\mu\text{g./100 ml.}$)	Plasma ($\mu\text{g./100 ml.}$)	Clearance (ml./min.)	Urine ($\mu\text{g./100 ml.}$)	Plasma ($\mu\text{g./100 ml.}$)	Clearance (ml./min.)
Males								
E.S.	25.6	0.94	11.6	12	0.9	24.6	5	4.7
R.E.	23.0	0.98	91	76	1.2	81	24	3.3
K.H.	23.4	0.95	70	131	0.5	148	43	3.2
A.W.	22.5	6.0	26	27.6	5.6	12	11	6.7
A.M.	23.5	3.1	15	24	2.0	11	8	4.4
E.K.	234	0.92	77	12	5.9	17	1.3	12
Mean	—	—	—	47	—	—	15	—
Females								
H.B.	33	4.6	15	46	1.5	13.6	15.2	4.1
J.W.	266	2.2	4.8	15.6	0.7	9.5	7.8	2.7
K.W.	253	5.8	26	4.7	32	9	2.8	19
E.B.	31.5	1.1	6.3	15	0.46	3.7	(1)	—
R.G.	31	6.1	2.5	31	0.5	4.8	27	1.1
Mean	—	—	—	22.5	—	—	13	—

Table 2. Androsterone and 3 α -hydroxy-5 β -androstan-17-one (aetiocholanolone) in glucuronide fractions from plasma and urine

	Androsterone			Aetiocholanolone		
	Urine ($\mu\text{g./100 ml.}$)	Plasma ($\mu\text{g./100 ml.}$)	Clearance (ml./min.)	Urine ($\mu\text{g./100 ml.}$)	Plasma ($\mu\text{g./100 ml.}$)	Clearance (ml./min.)
E.K.	259	2.1	118	241	1.3	177
J.W.	99	1.5	133	80	1.0	175
K.W.	33	2.0	113	50	2.5	137
Mean	—	1.9	—	—	1.6	—

Table 3. *Recovery of steroids from a mixture of pure steroids by chromatographic analysis in the apparatus described*

	Added ($\mu\text{g.}$)	Found ($\mu\text{g.}$)	Recovery (%)
3 β -Hydroxyandrost-5-en-17-one (dehydroepiandrosterone)	12.7	12.0	94
Androsterone	9.1	9.5	104
3 α -Hydroxy-5 β -androstan-17-one (aetiocholanolone)	13.9	14.2	102
3 α -Hydroxy-5 β -androstan-11:17-dione	6.5	4.8	74
3 α :11 β -Dihydroxy-5 β -androstan-17-one	5.4	4.5	83
Total	47.6	45.0	94

preparations used in the present work was inhibited by 0.03M-KH₂PO₄. Incubation of potassium dehydroepiandrosterone sulphate (74 $\mu\text{g.}$ of steroid) with the enzyme (35 000 units) in 0.5M-acetate buffer (pH 4.0, 25 ml.), under the same conditions as used in these studies, liberated 5% of the 17-oxo steroid (4 $\mu\text{g.}$) as benzene-extractable material. It has therefore been assumed that steroid sulphates in the amounts encountered are not attacked under the conditions described and that any material extracted by benzene after hydrolysis with the enzyme preparation has been liberated from conjugation as glucuronide. If this assumption is justified then the dehydroepiandrosterone glucuronide found in the large plasma samples (K.W. and E.K.) does not appear to have been excreted. No recognizable free 17-oxo steroids were found in material extracted from plasma by benzene before hydrolysis, even in the large samples.

A measure of the accuracy of the chromatographic analysis and determination procedures is given by the recoveries of five pure steroids (Table 3). The mean recovery of the three 11-deoxy-17-oxosteroids is 100%, and the highest error is 6%. Thus calculating the renal clearance values involving the ratio of two determinations the error may be as high as 13%. The recovery of 17-oxo steroids with an 11-oxygen function is less satisfactory.

DISCUSSION

The wide range of values for the dehydroepiandrosterone sulphate and androsterone sulphate concentrations in plasma from males was similar to that reported by Migeon (1956). Whereas in plasma from males the average androsterone sulphate concentration is approximately equal to that from females, in males the dehydroepiandrosterone sulphate concentration is more than twice that found in females. Migeon, who found no difference between the levels of plasma 17-oxo steroids in male and female subjects, states that increased values for 17-oxo steroid conjugates in the plasma of females are found midway through the menstrual cycle. The number of cases so far examined is not enough for any general conclusions to be drawn.

The renal clearance values of androsterone sulphate were, in ten out of eleven subjects, one to six times those for dehydroepiandrosterone sulphate, but the calculated reabsorption or retention of these compounds (Beaumont & Dodds, 1944) was greater than 90 and 95% respectively.

The clearance values for the glucuronides are comparable with that of inulin and suggest that these compounds may be excreted by glomerular filtration alone.

SUMMARY

1. In six male subjects and four female subjects peripheral plasma was found to contain the following 17-oxo steroids conjugated as sulphate: 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone), 12–131 $\mu\text{g.}/100\text{ ml.}$ and 5–46 $\mu\text{g.}/100\text{ ml.}$; androsterone, 1–43 $\mu\text{g.}/100\text{ ml.}$ and 1–27 $\mu\text{g.}/100\text{ ml.}$

2. In three larger samples of plasma, androsterone glucuronide (1.5–2.1 $\mu\text{g.}/100\text{ ml.}$) and 3 α -hydroxy-5 β -androstan-17-one (aetiocholanolone glucuronide; 1–2.5 $\mu\text{g.}/100\text{ ml.}$) were found. Small amounts of 3 α -hydroxy-5 β -androstan-11:17-dione and 3 α :11 β -dihydroxy-5 α -androstan-17-one were present, each on one occasion, in the glucuronide fraction of these larger samples.

3. Renal clearance values for dehydroepiandrosterone sulphate ranged from 0.5–6 ml./min. in ten out of eleven subjects. The corresponding values for androsterone sulphate were 1–12 ml./min.

4. Renal clearance values for androsterone and aetiocholanolone glucuronide ranged from 113 to 177 ml./min.

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REFERENCES

- Allen, W. M. (1950). *J. clin. Endocrin. Metab.* **10**, 71.
- Beaumont, G. E. & Dodds, E. C. (1944). *Recent Advances in Medicine*, 11th edn., p. 65. London: J. and A. Churchill Ltd.
- Bush, I. E. (1956). *Biochem. J.* **62**, 16P.

- Callow, N. H., Callow, R. K. & Emmens, C. W. (1938). *Biochem. J.* **32**, 1312.
- Clayton, G. W., Bongiovanni, A. M. & Padadatos, C. (1955). *J. clin. Endocrin. Metab.* **15**, 693.
- Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
- Edwards, R. W. G., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrin.* **2**, 53.
- Kellie, A. E. & Smith, E. R. (1956). *Nature, Lond.*, **176**, 323.
- Kellie, A. E. & Wade, A. P. (1957). *Biochem. J.* **66**, 196.
- Migeon, C. J. (1956). *J. biol. Chem.* **218**, 941.
- Migeon, C. J. & Plager, J. E. (1954). *J. biol. Chem.* **209**, 767.
- Romanoff, E. B., Hudson, P. & Pincus, G. (1953). *J. clin. Endocrin. Metab.* **13**, 1546.
- Roy, A. B. (1956). *Biochem. J.* **62**, 44.
- Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
- Wilson, H. (1954). *Arch. Biochem. Biophys.* **52**, 217.

A New Method for the Isolation of Deoxyribonucleic Acids: Evidence on the Nature of Bonds between Deoxyribonucleic Acid and Protein

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When homogenized mammalian tissue was extracted with a two-phase phenol-water mixture at room temperature ribonucleic acids (RNA) were released into the aqueous layer, whereas deoxyribonucleic acids (DNA) remained completely insoluble with the denatured protein. This method has been developed as a preparative procedure for RNA (Kirby, 1956*b*).

Replacement of the water by solutions of certain anions brought DNA as well as RNA into the aqueous layer and the effectiveness of some of the salts used has been reported in a preliminary note (Kirby, 1956*a*).

Most of the tests have been carried out with rat liver, but similar results have been obtained with rat kidney, spleen, testis, a hepatoma and calf thymus glands. The tissue was homogenized with isotonic solutions of the salts, adjusted to pH 6.5 in order to avoid complications by release of nuclear materials through osmotic or pH effects. DNA was liberated when potassium cyanide or ethylenediamine was used at pH 10, but remained insoluble if the pH was 6.5.

EXPERIMENTAL

Test for the release of deoxyribonucleic acids

The salt solutions were prepared either directly from the sodium salt or by weighing the acid, titrating it with *N*-NaOH to pH 6.5 and adjusting to the correct volume with water. When the sodium salt was insoluble at 0.15*M*-concentration, as with riboflavin phosphate, nitroso-2-naphthol-3:6-disulphonate (nitroso R salt) and spermine phosphate, a saturated solution was used. Complexing agents which were insoluble in water were dissolved in 1 or 2 ml. of 2-methoxyethanol, then water was added and the mixture used with any precipitate present.

Pieces of tissue (1.5 g.) were treated with the solution of the compound to be tested (20 ml.) in an all-glass tissue grinder and the mixture was poured off from any fibrous matter which remained at the bottom of the tube. Phenol [(90%, w/w); 20 ml.] was added to the tissue mixture and the two-phase system was stirred or shaken vigorously for 1 hr. at room temperature. The extract of phenol and water was then centrifuged at 1000 g for from 10 min. to 1 hr., depending upon the viscosity of the aqueous layer. A volume (1 ml.) of this aqueous supernatant layer was withdrawn. Dische's (1930) reagent (2 ml.) was added and the mixture was heated on a boiling-water bath. The development of a blue colour was a positive indication of DNA. Another 1 ml. portion of the aqueous layer was withdrawn, 1 ml. of ethanol added and the mixture shaken vigorously. A fibrous precipitate showed that DNA was present. The precipitation test was essential where the Dische colour was inhibited by the salt in solution, as for example with KSCN, $\text{Na}_2\text{S}_2\text{O}_3$ and *p*-aminosalicylate. The Dische test was then applied to the precipitate after washing and dissolution.

It was essential to homogenize the tissue in the salt solution first and then to treat with the phenol, since no DNA was released by treating tissues with water and phenol and subsequently adding the effective salts.

*Extraction of liver homogenates with *n*-butanol and protein denaturants*

Rat liver (7.5 g.) was homogenized in water (100 ml.); 20 ml. portions were taken and the various compounds shown in Table 1 added, and each was then shaken with *n*-butanol (20 ml.). When the liver was homogenized in 0.15*M*-sodium salicylate and treated with the same additives as above, exactly the same results were obtained.

Rat liver (1.5 g.) was homogenized in 20 ml. of 0.15*M*-sodium salicylate; resorcinol (10 g.) was added and the mixture shaken, but no DNA could be detected after removal of insoluble material by centrifuging. Addition of *n*-butanol (8 ml.) and *n*-hexylresorcinol (6 g.) with the same homogenate and subsequent separation resulted in a positive test for DNA being given by the aqueous layer.

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