SUMMARY

1. Rams were injected with [^{32}P]phosphate and [*carboxy*-^{14}C]stearic acid, and a study was made of the rate of appearance of labelled phosphorus compounds and fatty acids in the seminal plasma and sperm of serially collected samples of semen.

2. The maximum ^{32}P-labelling of seminal glycerylphosphorylcholine occurred some 15–18 days after injection, and next appeared the sperm phospholipids, acid-soluble phosphorus and a fraction designated as residual phosphorus, all having maxima at about 21–26 days. The sperm deoxyribonucleic acid appeared in a labelled form much later, reaching a maximum at 50–52 days.

3. In one animal ejaculating non-motile sperm the appearance of labelled glycerylphosphorylcholine in the semen was considerably delayed compared with normal rams.

4. In a ram in which the connexions between the epididymides and testes were severed surgically the seminal-plasma glycerylphosphorylcholine and sperm acid-soluble phosphorus became labelled with ^{32}P, but no appreciable activity appeared in the phospholipids, residual phosphorus and deoxyribonucleic acids of the ejaculated sperm.

5. Simultaneous injection of a ram with [^{32}P]-phosphate and [^{14}C]stearic acid did not indicate any transfer of labelled fatty acids to the sperm at the time of formation of glycerylphosphorylcholine.

6. From these results with isotopes an assessment has been made of the minimum time taken for the sperm to pass through the epididymides and also the time taken for the spermatocytes to be converted into the spermatozoa during spermatogenesis in the ram testes.

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Comparative Studies on the Liver Sulphatases

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Although the widespread distribution of the arylsulphatases is well known, there is little information available on the amounts of these enzymes present in the various species, the only attempt at comparative studies being that of Rutenburg & Seligman (1956) whose work is diminished in value because of the failure to appreciate the occurrence of several different sulphatases in their preparations. At least three arylsulphatases occur in mammalian tissues; sulphatases *A* and *B* (Roy, 1953*b*, 1954*a*) rapidly hydrolyse dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) but attack potassium *p*-nitrophenyl sulphate (NPS) only very slowly. These two enzymes may be separated by paper electrophoresis at pH 7, sulphatase *A* moving towards the anode

and sulphatase *B* towards the cathode. Sulphatase *C*, in striking contrast, hydrolyses NPS very rapidly and unlike the former two enzymes is extremely difficult to obtain in true solution (Roy, 1956*c*). The assay of the arylsulphatases in crude tissue preparations is further complicated by a number of factors, including the following: the destruction of the liberated phenol by the tissue (Dodgson & Spencer, 1953), the occurrence of an endogenous inhibitor in many tissues (Maengwyn-Davies & Friedenwald, 1954), the influence of many naturally occurring ions on the activities of the arylsulphatases (Roy, 1955, 1956*c*), the insolubility of sulphatase *C* (Roy, 1956*c*) and the highly anomalous kinetics of sulphatase *A* (Roy, 1953*b*). These factors make the assay of the arylsulphatases in unfractionated tissue preparations a matter of some difficulty.

A method has, however, been developed for the assay of the arylsulphatases in liver preparations. It depends upon the isolation of the usual cell fractions from a homogenate of the tissue, followed by the assay of each fraction for the various enzymes. The sum of these activities is taken as being the activity of the whole tissue. By this means many of the complicating factors listed above could be eliminated, or at least their effects made relatively constant. As already demonstrated (Roy, 1954*b*), isolated cell components do not destroy nitrocatechol; the concentration of sulphatase *C* in the microsomal fraction (Dodgson, Spencer & Thomas, 1954) and of sulphatases *A* and *B* in the mitochondrial and microsomal fractions (Roy, 1954*b*) allows the use of short incubation times, which in turn eliminates many of the complex kinetics shown by sulphatase *A* (Roy, 1957*b*). The preparation of the cell fractions under highly standardized conditions minimizes the effects of any endogenous inhibitors which may be present. Those occurring in the soluble fraction of the cell will accumulate in the final supernatant, which contains little sulphatase activity, and the amounts of those occurring in any of the particulate fractions will be kept constant by the standardized method of preparation.

This method has been applied to a study of the distribution of the arylsulphatases in the livers of various species and of changes in the arylsulphatase activity brought about by a number of experimental procedures. Determinations of steroid sulphatase activity (Roy, 1957*a*) and of the ability to synthesize dehydro*epi*androsterone sulphate (DHAS) (Roy, 1956*b*) were carried out at the same time.

EXPERIMENTAL

Preparation of nitrocatechol sulphate. The method used was that of Roy (1953*a*) with modifications similar to those of Dodgson & Spencer (1956) to remove the small amounts of nitropyrogallol disulphate which contaminated the original preparations (Roy & Kerr, 1956). The method is given here in detail because of the increasing use of NCS as a substrate for the assay of the arylsulphatases.

To 350 g. of KOH and 350 g. of potassium persulphate in 5 l. of water was added 150 g. of *p*-nitrophenol, and the mixture was stored at 37° for 48 hr. After bringing to pH 4 with H_2SO_4 the mixture was cooled to 5° and filtered, the precipitate being discarded. Phenols remaining in the filtrate were removed by extracting five times with 1 l. portions of ether. The aqueous phase was brought to pH 10–11 with KOH soln., left overnight at 5°, decanted from the underlying crystalline mass and concentrated *in vacuo* to about 750 ml. Acetone (2 vol.) was added and the precipitate of inorganic material filtered off. The filtrate was concentrated *in vacuo* until crystallization occurred. When crystallization was complete the crude NCS, as the potassium phenoxide, was filtered off and sucked dry at the pump. The filter cake was dissolved in boiling water and acetic acid added (1 ml./g. of filter cake) to precipitate the NCS in the phenolic form. This was twice recrystallized from water to remove the nitropyrogallol disulphate and then reconverted into the potassium phenoxide by crystallizing from the minimum volume of boiling water containing a 20% excess over the theoretical amount of KOH. The NCS was finally recrystallized from water. The usual yield is approximately 15 g. and another 4 g. may be obtained from the mother liquors of the last two crystallizations.

The particular crystalline form which is obtained depends upon the exact conditions of the crystallization. Rapid cooling and constant agitation of the hot saturated solution causes a monohydrate to appear as yellow needles from the still warm solution. Slow cooling without agitation causes orange prisms of a dihydrate to crystallize from the cold solution. Drying over P_2O_5 *in vacuo* at room temperature converts the dihydrate into the monohydrate and finally into the anhydrous form. The drying is slow and rather erratic, so that the anhydrous compound is best obtained by finally drying the material over P_2O_5 *in vacuo* at 60° for 4 hr. (Found: C, 23·1; H, 1·1. Calc. for $C_6H_3O_7NSK_2$: C, 23·1; H, 0·97%).

It should be noted that NCS, as the potassium phenoxide, explodes on heating, as do many simple phenoxides (Dr J. W. Minnis, personal communication). This behaviour necessitates the use of considerable care during elementary analyses.

Animal experiments. The animals used were stock animals from the various University departments. Partial hepatectomy in rats and mice was performed by the technique of Higgins & Anderson (1931). Necrosis of the liver in these species was induced by the subcutaneous injection of CCl_4 (5·3 g./kg.) as a 50% solution in olive oil (Levvy, Kerr & Campbell, 1948). In guinea pigs, scurvy was induced by feeding the ascorbic acid-deficient diet of Kodicek & Loewi (1955). Granuloma formation in guinea pigs was caused by the subcutaneous injection of a 1% solution of carrageenin (Robertson & Schwartz, 1953).

Cell fractionation. This was carried out by the standard technique of Hogeboom (1955), a 10% suspension of the liver in 0·25 M-sucrose being used as the starting material. The centrifugal forces used for separating the various cell fractions were: nuclei, 700 *g*; mitochondria, 5000 *g*; microsomes, 24 000 *g*. For assay, these fractions were sus-

pended in water to give a concentration corresponding to a 20% suspension of the liver and this was diluted with water as necessary (Table 1).

Enzyme assays. Sulphatases *A* and *B* together (sulphatase *A* + *B*) were determined with NCS as substrate in 0·13 M-acetate buffers. The general experimental procedures were as previously described (Roy, 1953*a*) and the exact conditions used for each species are summarized in Table 1. Sulphatase *C* was determined with NPS as substrate in 0·13 M-2-amino-2-hydroxymethylpropane-1:3-diol–acetic acid buffers (tris buffers). The general experimental conditions have already been described (Roy, 1956*c*) and the details are given in Table 1.

Steroid sulphatase was determined, as previously described (Roy, 1957*a*), with DHAS as substrate at pH 7·8 in tris buffer. Because of the small amounts of this enzyme present the optimum conditions for each of the species studied were not determined. Only the microsomal fraction was assayed as, at least in the rat (Roy, 1957*a*), steroid sulphatase activity is restricted to this fraction. The time of incubation was 3 hr. for rat and ox tissue; for all the other species incubation times of 17 hr. were required.

The ability to synthesize DHAS was determined, exactly as previously described (Roy, 1956*b*), with a second sample of liver (5 g.) to prepare the enzyme system. No attempt was made to determine the optimum conditions for the various species.

Paper electrophoresis. A 25% homogenate of the liver in water was prepared in a Potter–Elvehjem (1936) homogenizer and was centrifuged for 2 hr. at 24 000 *g* at 0°. A sample of the clear supernatant was removed, care being taken to avoid contamination both by the loosely packed sediment and by the upper layer of fatty material. Samples (0·1 ml.) of this material were then submitted to paper electrophoresis on Whatman no. 100 paper for 16 hr. at 200 v in 0·03 M-diethylbarbiturate buffer, pH 7·4. The paper was then cut into 1 cm. strips, which were incubated for an appropriate time (1–6 hr.) with nitrocatechol sulphate in acetate buffer. For sulphatases of the *A* type the conditions were 0·003 M-NCS at pH 5 in 0·13 M-acetate, and for the *B* type 0·03 M-NCS at pH 6 in 0·13 M-acetate. The amounts of nitrocatechol liberated were determined as usual (Roy, 1953*a*).

RESULTS

Fig. 1 shows that under the experimental conditions specified in Table 1 the amount of the phenol liberated is directly proportional to the concentration of the tissue in the reaction mixture.

Because of the complexity of the method a high degree of accuracy was not to be expected, but the results shown in Table 2 demonstrate it to be sufficiently reproducible for the present investigation. Similar experiments showed the validity of the method used in the assay of DHAS synthesis.

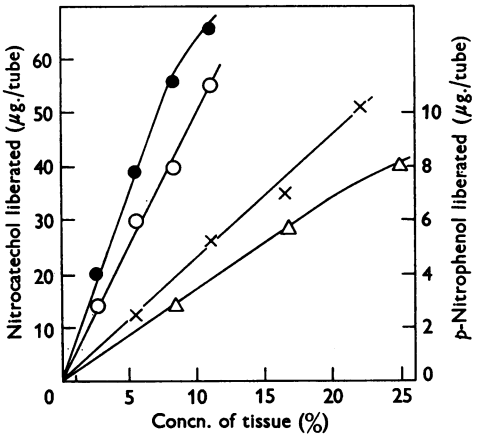

Fig. 1. Effect of tissue concentration on the rate of hydrolysis of sulphate esters. Volume of reaction mixture, 0·8 ml., containing 0·2 ml. of a suspension of the appropriate cell fraction equivalent to the concentration of whole tissue indicated. Substrate concentration and pH were as indicated in Table 1. Hydrolysis of NCS by rat-liver mitochondria, ○; by rat-liver microsomes, ●; by guinea-pig liver mitochondria, △. Hydrolysis of NPS by rat-liver microsomes, ×.

Table 1. *Conditions used in the assay of arylsulphatases in the livers of different species*

Volume of the reaction mixture was 0·8 ml., containing 0·2 ml. of tissue suspension corresponding to a 20% suspension of the liver except where otherwise specified. Incubation was for the time indicated.

	Sulphatase *A* + *B*			Sulphatase *C*		
Species	pH	Substrate concn. (mM)	Time (min.)	pH	Substrate concn. (mM)	Time (min.)
Rat	6·0*	30	15	7·7	4	15
Mouse	6·4†	30	15	8·3	5	60
Guinea pig	5·5	3	15	—	—	—
Rabbit	6·2	30	15	8·1	10	60
Ox	6·0	30	15	8·0	10	30
Hen	6·0	30	15	7·8	3	60
Frog	6·4†	30	15	—	—	—
Stickleback‡	6·0	30	15	—	—	—

* Tissue concentration: 5%. † Tissue concentration: 10%. ‡ Optimum conditions were not determined.

Comparative studies

The results of these experiments are summarized in Table 3. It should be noted that the units used to express the activity of steroid sulphatase and of the DHAS-synthesizing system differ from those used for the arylsulphatases by a factor of 10^{-3}.

Rat. The animals were of the Wistar strain, aged 3½–4 months. The most interesting result is the very pronounced sex difference in the activities of several of the enzymes. The sulphatase *C* and steroid sulphatase activities of the livers of male rats are about double those of the livers of female animals. These differences were highly significant ($P<0.001$). There was apparently a greater sulphatase $A+B$ activity in the livers of male rats, but this was not highly significant ($P<0.1$). As previously reported (Roy, 1956*b*), the ability to synthesize DHAS is very much greater in the livers of female rats than of male rats; this difference is again highly significant ($P<0.001$). Gonadectomy had little effect on these sex differences; even 8 weeks after the operation the only significant change was the fall in steroid sulphatase activity of the livers of castrated male animals.

In young rats, aged 2½ weeks, the sulphatase $A+B$ activity was extremely high; both sulphatase *C* and steroid sulphatase activities were slightly lower than normal.

Table 2. *Results of replicate analyses performed on single rat livers*

Samples 1–4 were obtained by treating independently four portions of one liver as described in the text. Samples 5–8 were obtained by fractionating four samples of a single homogenate of another liver. Results are expressed as μmoles of the phenol liberated/hr./g. of tissue under the conditions specified in Table 1.

Sample no.	Sulphatases $A+B$	Sulphatase *C*
1	195	19·6
2	211	19·2
3	248	21·5
4	196	21·7
Mean	213	20·5
5	195	18·4
6	200	23·4
7	186	21·7
8	193	21·0
Mean	194	21·1

Table 3. *Amounts of certain enzymes present in the livers of various species under different conditions*

Assays were carried out under the conditions listed in Table 1 and the results are expressed as μmoles of sulphate ester hydrolysed (the arylsulphatases), μm-moles of ester hydrolysed (steroid sulphatase) or synthesized (DHAS synthesis)/hr./g. of tissue in each case. Values quoted are the means, together with the standard deviations; the numbers of animals used are given in parentheses and details of the various treatments are given in the text. M, male; F, female; J, immature animals.

Species	Sex	Treatment	Sulphatases $A+B$	Sulphatases *C*	Sulphatases Steroid	DHAS synthesis
Rat	M	—	207±21 (8)	15·4±4·2 (8)	403±91 (8)	53±22 (20)
	M	Castration	208 (4)	12·1 (4)	279 (4)	72 (4)
	M	CCl_4	214±32 (8)	12·0±2·5 (8)	258±103 (8)	25±17 (8)
	F	—	187±20 (8)	8·0±1·2 (8)	187±62 (8)	149±37 (14)
	F	Castration	176 (4)	7·6 (4)	182 (4)	152 (4)
	F	Hepatectomy	297±22 (7)	8·9±2·0 (7)	177±74 (7)	135±25 (7)
	J	—	422 (4)	4·5 (4)	66 (2)	—
Mouse	M	—	55±14 (8)	1·5±0·4 (8)	64±11 (8)	7±4 (8)
	M	Hepatectomy	94 (4)	1·6 (4)	68 (4)	—
	F	—	35±16 (8)	1·6±0·4 (8)	52±19 (8)	34±18 (8)
	F	CCl_4	70±23 (8)	0·7±0·2 (8)	0 (8)	33 (3)
Guinea pig	M	—	52±12 (6)	0	0	72±9 (8)
	M	*Salmonella*	89±21 (5)	0	0	72±7 (5)
	M	Carrageenin	45 (4)	—	—	77 (4)
	M	Scorbutic animal	87 (4)	—	—	54 (4)
	F	—	38±7 (9)	0	0	78±11 (9)
	F	*Salmonella*	92±24 (6)	0	0	67±27 (6)
	F	Scorbutic animal	71 (2)	—	—	89 (2)
Rabbit	M	—	45 (2)	1·4 (2)	130 (1)	17 (1)
	F	—	61 (1)	1·7 (1)	106 (1)	18 (1)
Ox	M	—	29 (2)	15 (2)	300 (2)	19 (2)
Hen	F	—	26 (2)	2·3 (2)	†	8 (2)
Frog	M	—	160 (7)	0	0	†
Stickleback	—	—	11 (12)*	0	—	—

* Livers were bulked before fractionation. † Trace of activity was detected.

In regenerating liver, 4 days after partial hepatectomy, there was a definite ($P < 0.001$) increase in the activity of sulphatase $A + B$ but little change in any of the other sulphate enzymes. In necrotic liver, 36 hr. after the administration of CCl_4, there appeared to be a decrease in the activity of steroid sulphatase ($P < 0.02$) but there was no significant change in the levels of any of the other enzymes. Abbott & East (1949) claimed to have detected a fall in rat-liver arylsulphatase activity after administration of CCl_4 but it is unfortunately not possible from the few details published to decide which of the various arylsulphatases was in fact being studied by these authors.

The sex difference in the ability to synthesize DHAS would seem to lie in the presence of differing amounts of the appropriate sulphotransferase in the two sexes rather than in any difference in the ability to synthesize 'active sulphate' (Robbins & Lipmann, 1956), as there is no sex difference in the rate of synthesis of phenyl sulphate by similar liver preparations. The synthesis of phenyl sulphate was assayed under the conditions used for the assay of DHAS synthesis except that the substrate was mM-phenol. Four preparations from the livers of male rats synthesized phenyl sulphate at a mean rate of 147 ± 43 μm-moles/hr./g. wet wt., whereas the corresponding mean value for four preparations from female rats was 125 ± 15 μm-moles/hr./g. It is obvious that for these no sex difference exists. This is further evidence for the view (Gregory & Nose, 1957) that two independent sulphotransferases are involved in the synthesis of aryl and steroid sulphates.

Mouse. The animals were used at an age of 2½–3 months. The general level of all the sulphate enzymes in mouse liver was very much lower than that in the rat (Table 3). There was no convincing sex difference in any of the sulphatases, the apparent greater sulphatase $A + B$ activity in the male not being highly significant ($P < 0.05$). With the DHAS-synthesizing system, however, there was a highly significant sex difference ($P < 0.001$) comparable with that in the rat.

In regenerating liver, 4 days after partial hepatectomy, there was a considerable increase in the activity of sulphatases $A + B$. In necrotic liver, 36 hr. after the administration of CCl_4, there was a similar rise in the activity of sulphatases $A + B$ ($P < 0.01$), together with a fall in the level of sulphatase C activity ($P < 0.001$). No steroid sulphatase activity whatever could be detected in necrotic mouse liver. In neither case was there any change in the ability to synthesize DHAS.

Guinea pig. In this species neither sulphatase C nor steroid sulphatase could be detected, either in the microsomes or in preparations obtained by the method used to prepare sulphatase C from ox liver (Roy, 1956*c*). With prolonged periods of incubation (up to 24 hr.) the hydrolysis of NPS by cell fractions could be detected, but the activity of the mitochondria was greater than that of the microsomes, and in both the activity was greater at pH 6 than at pH 8, suggesting that enzymes of the sulphatase A or B types were involved. This is in agreement with the work of Morimoto (1937) and of Tanaka (1938), who found the pH optimum for the hydrolysis of NPS by guinea-pig liver to be in the region of pH 6.

As seen from the results in Table 3, there was no significant sex difference in either the level of sulphatases $A + B$ or the DHAS-synthesizing system.

During the period of the experiments a *Salmonella* infection occurred in the guinea-pig colony; the sulphatase activities of the livers of these infected animals were very much higher than normal ($P < 0.01$). Unfortunately the causative organism was not identified, but it was almost certainly *Salmonella typhimurium*. The sulphatase activity of the livers of scorbutic guinea pigs also seemed to be rather higher than normal. In animals laying down connective tissue in response to carrageenin injection 4 days previously no significant differences in the levels of these enzymes could be detected.

Other species. Results obtained with the other species studied are summarized in Table 3. The main point to note is that sulphatase C could not be detected in the livers of the frog (*Rana temporaria*) and of the stickleback (*Gasterosteus aculeatus*). Steroid sulphatase was also lacking from the former species.

Occurrence of sulphatases A *and* B

It has already been shown that there are at least two soluble sulphatases, sulphatases A and B, in the livers of the ox and the rat (Roy, 1953*a*, 1954*a*), and it was of interest to study these in other species. From the shapes of the pH–activity curves, shown in Fig. 2, it seemed likely that enzymes corresponding to both sulphatases A and B were present in all the species investigated, with the possible exception of the frog, as those curves were analogous to those previously obtained (Roy, 1954*a*) in an investigation of ox liver, known to contain both sulphatases A and B. The pH curve of the sulphatase of stickleback liver was similar to that of the frog.

The results of experiments designed to separate sulphatases A and B by paper electrophoresis are summarized in Fig. 3. Enzymes corresponding to sulphatases A and B were detected in all the species studied except the guinea pig, the hen, the frog and the stickleback. In the frog the arylsulphatase seemed to be a pure B type of enzyme,

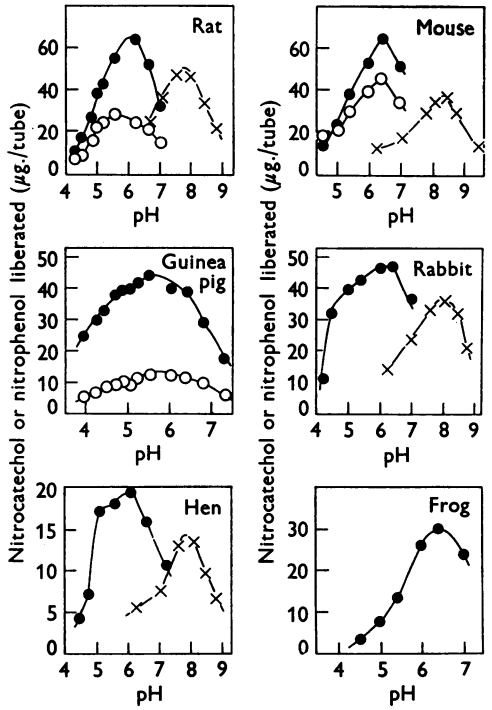

Fig. 2. Effect of pH on the rate of hydrolysis of NCS and NPS by cell fractions in various species. Conditions were as specified in Table 1, apart from variations in pH. Hydrolysis of NCS by mitochondria, ●; by microsomes, ○. Hydrolysis of NPS by microsomes, ×.

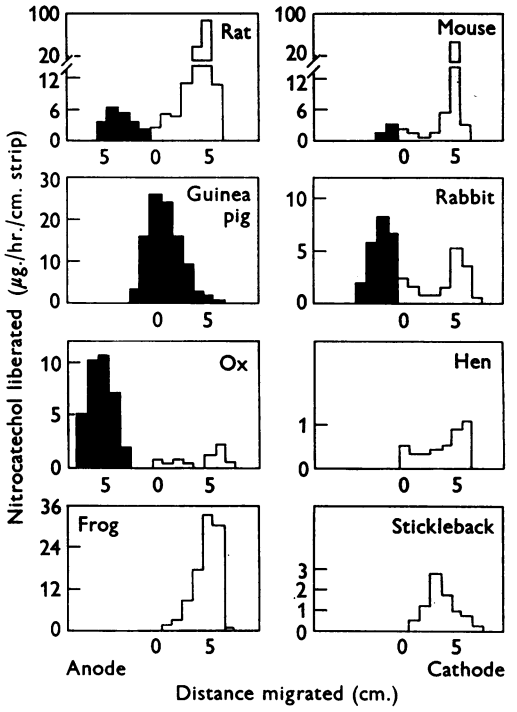

Fig. 3. Electrophoretic separation of the soluble sulphatases of various species. Enzyme preparations were obtained as described in text. Run for 16 hr. at pH 7·4 in 0·03 M-diethylbarbiturate buffer. Assays were carried out at pH 5 in 3 mM-NCS (black areas) and at pH 6 in 0·03 M-NCS (clear areas).

as shown by its behaviour on electrophoresis (Fig. 3), by its pH curve (Fig. 2) and by its substrate concentration–activity curve (Fig. 4), the latter giving a value for the K_m in the region of 0·01 M-NCS, a value comparable with that of a mammalian type *B* arylsulphatase. The situation in the stickleback was apparently identical with that in the frog. In the guinea pig the situation was quite different; although only one arylsulphatase could be detected by paper electrophoresis (Fig. 3), the shape of the pH curve (Fig. 2) suggested that more than one such enzyme was present. Attempts to prepare two enzymes by the methods used to obtain ox-liver sulphatases *A* and *B* (Roy, 1953*b*, 1954*a*) were not successful, but the results were consistent with more than one arylsulphatase being present. In the hen also the pH curve (Fig. 2) would suggest that both sulphatases *A* and *B* were present, although they could not be detected by electrophoresis (Fig. 3). However, the arylsulphatases of the hen were apparently much less stable during the electrophoresis than were those of the other species studied, as the total amount of activity recovered after electrophoresis was only a very small proportion of that originally

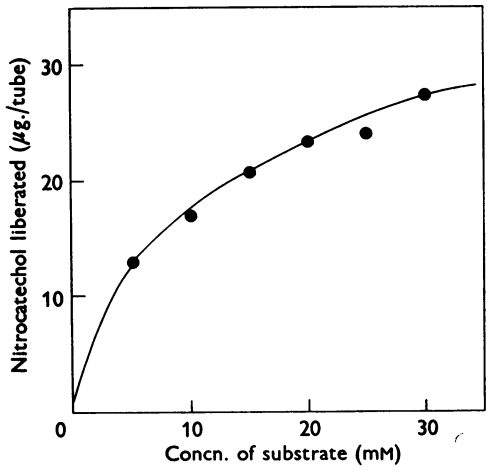

Fig. 4. Effect of variations in substrate concentration on the hydrolysis of NCS by frog-liver mitochondria at pH 6·4.

Table 4. *Intracellular distribution of the aryl sulphatases*

Assays were carried out as listed in Table 1. Numbers of animals were as shown in Table 3. Figures are percentage of total recovery found in each fraction. N, Nuclei; M, mitochondria; P, microsomes; S, supernatant.

		Sulphatases $A+B$				Sulphatase C			
Species	Sex	N	M	P	S	N	M	P	S
Rat	M	7±1	45±4	32±5	19±4	5±4	8±8	88±8	0
	F	8±1	44±6	29±3	19±4	0	0	100	0
Mouse	M	7±1	42±4	29±2	21±4	0	0	100	0
	F	13±9	46±8	21±5	20±15	0	0	100	0
	F*	10±2	31±1	27±1	32±2	0	0	100	0
Guinea pig	M	8±2	38±8	12±2	43±8	—	—	—	—
	F	11±3	45±5	13±3	33±5	—	—	—	—
Rabbit	M	15	34	16	36	0	0	100	0
	F	15	32	14	39	0	0	100	0
Ox	M	14	47	20	20	13	5	66	18
Hen	F	8	27	47	19	11	27	63	0
Frog	M	12	26	10	52	—	—	—	—
Stickleback	—	23	58	19	0	—	—	—	—

* Liver necrosis was induced by CCl_4.

present. This instability might well account for the apparent absence of a sulphatase *A* from hen liver.

The ratio of sulphatase *A* to sulphatase *B*, as determined by paper electrophoresis, varies considerably in the different species. The ratio of sulphatase *A* to sulphatase *B* activity ranges from 0·1 in the rat and the mouse through 2 in the rabbit to 6·5 in the ox. These values are only approximate, as the method cannot take into account the differing stability of the two enzymes to paper electrophoresis, nor the complicating effects of the anomalous kinetics of sulphatase *A*, which become important at the high dilutions obtained after electrophoretic separation of the enzymes.

Intracellular localization of the arylsulphatases

It has previously been claimed that, in both the mouse and the rat (Roy, 1953*a*, 1954*b*), the greater part of the sulphatase *A* and *B* activity is associated with the mitochondria. These claims are supported by the results shown in Table 4. The distributions found in the other species are in general agreement with the original observations, although in the guinea pig, and to an even greater extent in the frog, a high proportion of the sulphatase $A+B$ activity occurs in the soluble fraction. It might well be that this does not reflect a fundamental change in the intracellular distribution of these enzymes in those species, but rather that their sulphatase-containing granules are more readily disrupted, so liberating the enzymes in a soluble form during the isolation procedure.

The solubility of the sulphatase $A+B$ activity of the mitochondrial and microsomal fractions is shown in Table 5. In this connexion a soluble

Table 5. *Solubility of sulphatases* A *and* B *in mitochondria and microsomes*

A 'soluble' enzyme is defined as one not sedimentable in 1 hr. at 24 000 *g*. Values are expressed as percentage of whole fraction.

Fraction		Rat	Mouse
Mitochondria	Total	100	100
	Soluble (*a*)	79	75
	Insoluble (*b*)	35	32
	$a+b$	114	107
Microsomes	Total	100	100
	Soluble (*a*)	33	26
	Insoluble (*b*)	52	61
	$a+b$	85	87

enzyme is defined as being one which is not sedimented on centrifuging the suspension of the particles in water for 1 hr. at 24 000 *g* at 0°.

Results shown for sulphatase *C* in Table 4 confirm the observations of Dodgson, Spencer & Thomas (1954) and show this enzyme to have a purely microsomal origin. The amounts of sulphatase *C* occurring in fractions other than the microsomes are simply those to be expected from contamination of the various cell fractions by the former, with a possible exception for the hen, although the considerable amounts of lipid present in the liver of this species may have interfered with the fractionation. It should be noted that in those cases where sulphatase *C* was apparently restricted entirely to the microsomes some activity could be found in the other cell fractions by using longer periods of incubation than normal.

The various treatments listed in Table 3 had no significant effect on the intracellular distribution of the various sulphatases except for the administration

of CCl_4 to mice, which increased the proportion of the sulphatase $A+B$ activity found in the soluble fraction, with a corresponding decrease in the amount present in the mitochondria. This effect could well be due to the toxic action of the CCl_4 making the cell particles more readily lysed (Dianzani, 1957).

The significance of the cell fractions obtained from the livers of the lower vertebrates has not been investigated. These fractions may be only distantly related to the corresponding fractions of mammalian liver, but this is of little significance in the present connexion. A consideration of the results shown in Table 4, however, suggests that the cell fractions obtained from the livers of the lower vertebrates are directly comparable with those obtained from mammalian liver.

DISCUSSION

As shown by the results summarized in Table 2 the method would seem to be a useful one for the determination of sulphatases in liver tissue, the only serious disadvantage being the tedious nature of the procedure. It would seem also that the method could be adapted without difficulty to any other tissue from which the appropriate cell fractions can be prepared. At present there is no way of assessing the absolute significance of the results, at least with regard to sulphatases *A* and *B*, as it is impossible to determine these enzymes in unfractionated preparations. With sulphatase *C*, however, the results for the rat are of the same order as those obtained by Dodgson, Spencer & Thomas (1953) with potassium *p*-acetylphenyl sulphate as substrate with unfractionated-liver preparations.

The results of the comparative studies need little comment. Sulphatases of the type of ox-liver sulphatase *B* (Roy, 1954*a*) have been found in all the vertebrate species studied, and these may be accompanied in the higher species by sulphatases comparable with ox-liver sulphatases *A* and *C* (Roy, 1953*b*, 1956*c*). Only in the guinea pig does a sulphatase comparable, at least in some respects, with sulphatase *A* occur without the simultaneous occurrence of a sulphatase *C*. Although sulphatases of the *A* and *B* type seem to be of ubiquitous occurrence, at least in the higher vertebrates, the results summarized in Fig. 3 show that there can be very considerable variations in the proportions of these two enzymes. The two extremes seem to be the rat and the ox; in the former a sulphatase of the *B* type accounts for by far the greater part of the sulphatase $A+B$ activity, whereas in the latter a sulphatase *A* is the predominant enzyme. In the guinea pig the sulphatases hydrolysing NCS, and presumably of the *A* or *B* types, appear to differ from the corresponding enzymes in all the other species studied; this is reflected not only by their behaviour on electrophoresis (Fig. 3) but also by the position of their optimum substrate concentration (Table 1).

Those species which are lacking in sulphatase *C* also seem to be deficient in steroid sulphatase. As shown by the results in Table 3 there seems to be a very close relationship indeed between these two enzymes. It has already been suggested (Roy, 1957*a*) on indirect evidence that these two activities are due to separate enzymes, but until direct evidence is obtained the possibility that only one enzyme is responsible for both types of activity cannot be ignored.

It is interesting to compare the situation in the vertebrates with that in the few invertebrate species so far studied. Electrophoretic (Roy, 1956*a*) and kinetic (Dodgson, Lewis & Spencer, 1954; Roy, 1956*a*) studies have shown that the arylsulphatase of the limpet, *Patella vulgata*, is comparable in many respects with a mammalian sulphatase of the *A* type. Similarly in the snail, *Helix pomatia*, the arylsulphatase would seem to be of the *A* type, to judge by its kinetic properties and its behaviour on electrophoresis (Jarrige & Henry, 1952). Again, in the locusts *Locusta migratoria* and *Schistocerca gregaria* the arylsulphatase seemed to be of an *A* type (Robinson, Smith & Williams, 1954). Dodgson, Lewis & Spencer (1954) did not detect in the limpet any sulphatase of the *C* type under conditions in which its presence should have been obvious had it occurred in significant amounts. In the few invertebrates so far studied therefore the sole arylsulphatase present would seem to be of the type of mammalian sulphatase *A*, in rather striking contrast with the situation in the lower vertebrates where the arylsulphatase is of the *B* type. It should be noted, however, that the invertebrate sulphatases do not show the kinetic anomalies of mammalian sulphatases of the *A* type. Another striking difference in the invertebrate sulphatases is seen in the steroid sulphatase; in mammals this enzyme is insoluble and has a pH optimum in the region of 8; in the mollusc it has a pH optimum of approximately 5 and is readily soluble (Roy, 1956*a*).

The changes in the levels of the sulphate enzymes under various experimental conditions are listed in Table 3 and little of a general nature can be said. The different behaviour of the sulphatases in the livers of rats and mice poisoned with carbon tetrachloride may well be due to the differing responses of these species to that substance: histological examination of the livers of animals treated with carbon tetrachloride showed that the amount of tissue damage was considerably less in the rat than in the mouse. This could well be the reason for the

differing responses in the two species. It seems likely that the rise in the sulphatase activity in the livers of scorbutic guinea pigs was due to the general changes induced in liver by ascorbic acid deficiency (Christian, 1947) rather than to any specific effect on the sulphatases. The only general conclusion which it seems possible to draw from this result is that sulphatases *A* and *B* (either or both) are present in increased amounts under conditions in which cell proliferation is occurring. There is also apparently a tendency for sulphatase *C*, and the associated steroid sulphatase, to be low in necrotic liver.

It is interesting to note the almost exact parallelism in the responses of sulphatases *A* + *B* and of β-glucuronidase (Levvy *et al.* 1948) in mouse liver to partial hepatectomy and to the administration of carbon tetrachloride. In the rat the parallelism was not so exact, but in this species the behaviour of β-glucuronidase is complicated by the presence of an endogenous inhibitor and also by the important strain differences between Wistar and Lister rats (Walker & Levvy, 1953). Such a close relationship between β-glucuronidase and sulphatases *A* and *B* is perhaps not unexpected if, as is discussed below, both enzymes occur in the lysosomes.

The results confirm the earlier claims (Dodgson, Spencer & Thomas, 1954; Gianetto & Viala, 1955) that sulphatase *C* is of purely microsomal origin and are also in agreement with the previous observations (Roy, 1953*a*, 1954*b*) that in the mouse and the rat the highest proportion of sulphatases *A* and *B* is localized in the mitochondrial fraction, although considerable amounts may be found in the other cell components. These results are apparently consistent with the claim that sulphatases *A* and *B* occur in the lysosomes (Viala & Gianetto, 1955), as these particles distribute themselves between the mitochondrial and microsomal fractions obtained by the usual procedure (Applemans, Wattiaux & de Duve, 1955). The present results are not in complete agreement with this view, however, as according to Viala & Gianetto (1955) when lysosomes are suspended in water all the associated sulphatase activity is liberated in a soluble form. As seen from Table 5 this is not the case. A considerable proportion of these sulphatases remains insoluble when the mitochondria, and more especially the microsomes, are treated with water. This is obviously not in accord with the views of Viala & Gianetto (1955) and it suggests that the sulphatases *A* and *B* of these two cell fractions are 'bound' in different ways. Again there is the observation (Roy, 1956*c*) that in the ox some of the sulphatases of this type are very closely associated with sulphatase *C* and remain insoluble, despite prolonged treatment with water: this would again suggest that there may be more than one form of sulphatases *A* and *B* in liver. The detailed description of the intracellular distribution of these enzymes is therefore still in doubt although the general picture is certainly that summarized in Table 4. It would seem reasonable to conclude that in all probability the view that sulphatases *A* and *B* are associated with the lysosomes is partly correct, but the possibility that some of these enzymes are associated with the true mitochondria, or more likely with the true microsomes, cannot be excluded.

Perhaps the most important general conclusion to be drawn from the work is that, apart from the possible interdependence of sulphatase *C* and steroid sulphatase, each of these enzymes can vary quite independently of one another, suggesting that each of them has a specific role to play in the metabolic processes of the animal. No conclusion about the function of the arylsulphatases can be drawn and consideration of the fact that one or more of these enzymes may be lacking from some species suggests that they cannot be fulfilling any very important role in the normal animal. The function of sulphatases *A* and *B* must be considered in relation to the function of the many other hydrolases which occur in the lysosomes; de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) have suggested that they play some role in localized intracellular digestion, but there is no evidence for, or against, this hypothesis.

SUMMARY

1. To minimize the difficulties inherent in the assay of the arylsulphatases in unfractionated tissue preparations these enzymes were determined in liver-cell fractions obtained from a sucrose homogenate of the tissue. Sulphatases *A* and *B* were together determined with nitrocatechol sulphate as substrate and sulphatase *C* was assayed with *p*-nitrophenyl sulphate as substrate.

2. Values are given for the arylsulphatase activities of the livers of several species. Steroid sulphatase and the steroid sulphate-synthesizing system were also assayed and the values are reported.

3. Sulphatase *C* and steroid sulphatase activities were much higher in the livers of male than of female rats. The level of the steroid sulphate-synthesizing system was considerably higher in the livers of female rats and mice than in the corresponding male animals.

4. The changes in these activities caused by various experimental procedures are described. The level of sulphatases *A* and *B* appeared to be elevated in proliferating tissues.

5. The approximate relative proportions of

sulphatases *A* and *B* were determined by paper electrophoresis. There were considerable variations in this ratio and sulphatase *A* appeared to be lacking in the lower vertebrates.

6. Sulphatases *A* and *B* were localized predominantly in the mitochondrial and microsomal fractions. Sulphatase *C* occurred only in the microsomes.

7. Although the distribution of sulphatases *A* and *B* was consistent with them occurring in the lysosomes they could not be completely extracted from the mitochondrial and microsomal fractions with water, so that it is probable that not all of these enzymes are localized in the lysosomes.

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The Biosynthesis of Penicillin

8. INVESTIGATION OF CYCLIC CYSTEINYLVALINE PEPTIDES AS PRECURSORS*

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The demonstration of the intact incorporation of L-cystine, presumably after reduction to cysteine (Arnstein & Grant, 1954*a*, *b*; Arnstein & Crawhall, 1957) and of the utilization of the carbon chain of valine for the biosynthesis of the β-lactam–thiazolidine ring system of penicillin (I) (Arnstein & Grant, 1954*a*; Stevens, Vohra & De Long, 1954; Arnstein & Clubb, 1957) has led to speculation on the mechanism by which these amino acids combine, and the nature of the more immediate precursors of the penicillin molecule so formed.

Although the utilization of valine nitrogen by

* Part 7. Arnstein & Margreiter (1958).

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