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A Comparison of Sodium Salicylate and 2:4-Dinitrophenol as Metabolic Stimulants *in vitro*

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Sodium salicylate as a peripheral metabolic stimulant exhibits the outstanding pharmacological feature of 2:4-dinitrophenol (DNP) and related compounds (Sproull, 1954*b*). DNP inhibits oxidative phosphorylation (Loomis & Lipmann, 1948), which is considered to be the source of its metabolic stimulant property (see Simon, 1953, for review). Brody (1956) has shown that salicylate also inhibits oxidative phosphorylation. The similarity of salicylate and DNP, although contrary to therapeutic and toxicological experience of these agents in man, is of great interest from the viewpoint of therapeutics, and suggests that the actions of the two compounds, as metabolic stimulants, may be identical. Some comparisons were made in the present investigation of the effects of salicylate and DNP on the respiration of tissue slices, in the hope of obtaining evidence which would permit a definite conclusion regarding difference or identity of the drugs' primary actions.

Peiss & Field (1952) found that DNP increased the metabolic rate of rat-brain slices with glucose or pyruvate as substrate, but not when succinate or α-oxoglutarate was employed. The first part of the present investigation was to determine whether this property of DNP is possessed by salicylate.

The second part of this investigation was to compare the concentration–response curves of the two compounds on mouse-liver slices, and to study the combined action of the compounds on this preparation.

METHODS

Brain slices. The rate of O_2 consumption of brain slices from fed male Wistar albino rats was determined by the direct method in Warburg respirometers at 37°. The slices, nominally 0·3 mm. thick, were prepared in a mechanical chopper and were separated and selected individually before being placed in the Warburg flasks; the medium was Krebs–Ringer phosphate, pH 7·4 (Krebs & Henseleit, 1932). The results were expressed as μl. of O_2/hr./mg. final dry wt. of tissue (Q_{O_2}). For each rat the Q_{O_2} values of the control and treated tissues were simultaneously determined in runs of 30 min. duration. Sodium pyruvate was prepared by neutralization of an iced ethanolic solution of redistilled pyruvic acid, and was recrystallized twice; α-oxoglutaric acid, m.p. 112°, was obtained commercially and was neutralized in aqueous solution immediately before use; substrate concentration was generally 0·01 M.

Liver slices. These were prepared from 3- to 8-month-old fed CBA mice, an improved chopper (Sproull, 1956) being used. The details of the manometric technique were as described above, with the exception that the mouse liver was transferred directly from the chopper to the flask without preliminary washing and separation of the slices. Glucose (0·011 M) was incorporated into the medium. The rate of O_2 uptake of the liver slices was, as usual, referred to the dry weight of the tissue after the run.

RESULTS

Rat brain

These experiments were to determine whether or not some concentration of sodium salicylate alters the metabolic rate of the tissue slices under certain experimental conditions. For a given concentration of sodium salicylate and a given substrate the hypothesis that the mean difference between the values of Q_{O_2} for the control and treated tissue (ΔQ_{O_2}) was zero (H_0) was tested sequentially against the alternative hypothesis that it differed from zero (H_1), with the sequential test of Student's hypothesis proposed by Wald (1947) and the tables of Arnold (1951). In each case, where the mean ΔQ_{O_2} was μ and the variance σ^2, it was decided whether $|\mu| < 2\sigma$ (i.e. H_0) or whether $|\mu| \geqslant 2\sigma$ (i.e. H_1). The maximum probability assigned to a decision in favour of

either hypothesis when, in fact, the other was true was 0·05. If H_1 was accepted, the 95 % confidence limits of the mean ΔQ_{O_2} were determined; provided zero was not included within the limits the sign of the mean ΔQ_{O_2} was thus formally established.

With mM-sodium salicylate, and glucose or pyruvate as substrate, a marked increase in oxygen consumption was found; this concentration of salicylate stimulates the metabolic rate of mouse-liver slices (Sproull, 1954*b*). On the other hand, when succinate or α-oxoglutarate was the substrate, mM-salicylate decreased the Q_{O_2} of the brain slices; there was no evidence of metabolic stimulation at lower concentrations of the drug, chosen in geometric progression. These results are summarized in Table 1. It was concluded that, if the form of the concentration–response curves is the same as previously found for mouse liver, salicylate has no appreciable stimulant action on the oxygen uptake of rat-brain slices when succinate or α-oxoglutarate is the substrate.

The possibility must be considered that the effect of mM-salicylate, when glucose was the substrate, may be the resultant of a substantial stimulation and a moderate depression. The mean ΔQ_{O_2} of rat-brain slices in glucose–Ringer with mM-salicylate was found to be +1·84; at 1·59 mM it was +2·35, and at $6{\cdot}31 \times 10^{-4}$ M +1·16. These figures are each the mean of eight observations, and approximate to those previously found for mouse-liver slices (Sproull, 1954*b*). An analysis of variance of these results is presented in Table 2; there was no significant departure from linear regression of ΔQ_{O_2} on log concentration, and therefore, since the regression coefficient is positive, unless the form of the concentration–response curve differs from that of mouse-liver slices, which as salicylate concentration increased showed increasing stimulation followed by depression (Sproull, 1954*b*), the effect of mM-sodium salicylate is solely stimulation, and not the resultant of a stimulation and a depression of metabolic rate.

Mouse liver

The present study was confined to the stimulation of oxygen consumption; only the first parts of the concentration–response relationships of salicylate and DNP on mouse-liver slices were investigated.

Table 1. *Effect of sodium salicylate on the rate of oxygen consumption of rat-brain slices in the presence of various substrates*

$\Delta Q_{O_2} = Q_{O_2}$ (control tissue) $- Q_{O_2}$ (treated tissue). The last column shows the conclusion drawn from the sequential test. (H_0 is the hypothesis that the mean ΔQ_{O_2} is zero, H_1 that it differs from zero.) The probability of accepting the wrong hypothesis is 0·05.

Substrate	Sodium salicylate concn. (M)	No. of runs required for termination of test	Mean control Q_{O_2}	Mean ΔQ_{O_2}	±95% confidence limits of mean ΔQ_{O_2}	Hypothesis accepted
Glucose (0·011 M)	10^{-3}	6	−10·0	+1·8	±0·53	H_1
Sodium pyruvate (0·01 M)	10^{-3}	6	−10·5	+1·6	±0·62	H_1
Sodium succinate (0·01 M)	10^{-3}	7	−10·3	−1·1	±0·66	H_1
	$6{\cdot}31 \times 10^{-4}$	9	−10·2	−0·5	—	H_0
	$3{\cdot}98 \times 10^{-4}$	5	− 9·9	+0·15	—	H_0
	$2{\cdot}51 \times 10^{-4}$	4	−10·0	+0·1	—	H_0
Sodium α-oxo-glutarate (0·01 M)	10^{-3}	10	− 8·0	−0·7	±0·30	H_1
	$6{\cdot}31 \times 10^{-4}$	8	− 7·8	−0·4	—	H_0
	$3{\cdot}98 \times 10^{-4}$	6	− 7·7	+0·1	—	H_0
	$2{\cdot}51 \times 10^{-4}$	5	− 7·7	−0·05	—	H_0

Table 2. *Analysis of variance of ΔQ_{O_2} of rat-brain slices in glucose–Ringer at three concentrations of sodium salicylate*

$\Delta Q_{O_2} = Q_{O_2}$ (control tissue) $- Q_{O_2}$ (treated tissue). Sodium salicylate concentrations (with mean ΔQ_{O_2} values in parentheses): $6{\cdot}31 \times 10^{-4}$ M (+1·16); mM (+1·84); 1·59 mM (+2·35).

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Variance between concentrations:			
Due to linear regression of ΔQ_{O_2} on log concn.	5·70	1	5·70
Deviation from regression	0·04	1	0·04
Variance within concentrations	9·91	21	0·47
Total	15·65	23	—

The maximum concentrations chosen were therefore sodium salicylate 2 mM and DNP 5×10^{-5} M; the lowest concentrations, arbitrarily selected, were $3{\cdot}5 \times 10^{-4}$ M and $8{\cdot}8 \times 10^{-6}$ M respectively.

Previous results (Sproull, 1954*b*) did not show a significant difference between the sexes in the response of mouse-liver slices to salicylate, but the results were not decisive. The substantial current improvement in technique, due mainly to the use of a reliable tissue chopper, improved the prospects of investigating this issue. Accordingly the sexes were considered separately. For the males four, and for the females two, intermediate concentrations were chosen, to give in each instance a succession of concentrations in geometric progression.

The results are shown in Fig. 1 and Tables 3 and 4. Before proceeding to the analyses of variance the homoscedasticity of the four sets of data was confirmed by Bartlett's test; in each instance $0{\cdot}50 > P > 0{\cdot}10$. In each case a linear relation, from which there was no significant departure, was established between ΔQ_{O_2} and logarithm of drug concentration. The regression coefficients of ΔQ_{O_2} on ln (concentration) were: salicylate, +1·53 (male), +0·95 (female); DNP, +2·39 (male), +1·46 (female). The significance of the differences between these regression coefficients was determined by the *t* test; the difference between the male salicylate and male DNP regression coefficients is highly significant, $P < 0{\cdot}001$; the corresponding value for the females is $0{\cdot}05 > P > 0{\cdot}02$. With each drug a significant sex difference in regression coefficients was established: for DNP, $P < 0{\cdot}001$; for salicylate $0{\cdot}01 > P > 0{\cdot}001$.

The preceding data permit a quantitative study of the combined action of salicylate and DNP on mouse-liver slices. This was undertaken as described by Gaddum (1948). The effect of a mixture of half each of equally effective concentrations of DNP and salicylate was compared with the equal actions of the individual drugs. Table 5 summarizes the results of these experiments. In both males and females the hypothesis that the efficacy of the mixture was equal to that of the individual drugs was rejected. Potentiation was found. This finding is not consistent with the possibility that salicylate and DNP have identical actions as metabolic stimulants. Furthermore, it also rules out the possibility of independent actions, since in each case the sum of the expected responses of the half concentrations is less than the critical ΔQ_{O_2} value (Fig. 1), i.e. +2·5 for the male series and +1·5 for the female series.

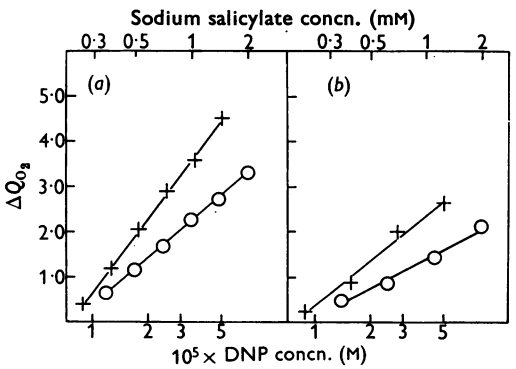

Fig. 1. Relation between concentration of sodium salicylate or 2:4-dinitrophenol, and increase in oxygen consumption, by (*a*) male and (*b*) female mouse-liver slices at 37° in glucose–Ringer phosphate, pH 7·4. Points represent the mean differences found, and the lines are drawn in accordance with the regression equations: +, DNP; O, sodium salicylate.

DISCUSSION

The first part of the present investigation represents an empirical comparison; full interpretation of the results is not possible at present. These results correspond with those obtained by Peiss & Field (1952) with DNP. The effective concentrations of salicylate were of the same order as previously found for mouse-liver slices (Sproull, 1954*b*), and the sensitivity of rat-brain slices to salicylate in Krebs–Ringer phosphate was much the same as that of mouse liver under the same experimental conditions, in contrast with the relative insensitivity of rat-brain mitochondria found by Brody (1956).

The conclusion that salicylate has no stimulant action when succinate or α-oxoglutarate was the substrate is governed by the arbitrary conditions laid down beforehand: the critical stimulant action chosen was one where mean $\Delta Q_{O_2} > 2\sigma$ over a range of concentration C_1 to C_2, such that $C_2/C_1 > \sqrt[5]{10}$. Smaller effects, or those over a lesser range of concentration, were disregarded as being trivial. Two further restrictions are, of course, the possibilities of an error of the second kind, or of a stimulant action having a threshold concentration equal to or greater than that of the depression of oxygen uptake.

The results obtained from the experiments on mouse-liver slices are more decisive. It has been established that there is no significant departure from linearity by any of the four log concentration–response curves. This is of interest, but it should be borne in mind that the data refer in each case to an unspecified part of the whole concentration–response curve. In contrast with the present findings, Alexander & Johnson (1956) found that a simple linear relation between serum concentration of salicylate and metabolic rate under basal conditions adequately accounted for their results

from human subjects. With mouse liver, for each sex the gradients of the concentration–response curves of salicylate and DNP are significantly different. This is regarded as suggesting difference in the primary actions of salicylate and DNP as metabolic stimulants *in vitro*. Taking the magnitude of the regression coefficients as a criterion, it is concluded that DNP is a more effective stimulant *in vitro* than salicylate.

Can the difference between the two drugs be expressed as a constant, regardless of sex? This question is posed rather than answered by the present results, but it is noted that the ratios of gradients of the log concentration–response curves are almost equal for both sexes; the gradients of the male curves are: DNP +2·39, salicylate +1·46 (ratio 1·64); for the females the corresponding figures are +1·53, +0·95 and the ratio is 1·61. This

Table 3. *Effect of sodium salicylate and 2:4-dinitrophenol on the rate of oxygen consumption of male and female mouse-liver slices*

$\Delta Q_{O_2} = Q_{O_2}$ (control) $- Q_{O_2}$ (treated tissue).

Sex	DNP concn. (M)	Mean $\Delta Q_{O_2} \pm 95\%$ fiducial limits	Sodium salicylate concn. (M)	Mean $\Delta Q_{O_2} \pm 95\%$ fiducial limits
Male	$8{\cdot}8 \times 10^{-6}$	$+0{\cdot}32 \pm 0{\cdot}35$	$3{\cdot}54 \times 10^{-4}$	$+0{\cdot}66 \pm 0{\cdot}35$
	$1{\cdot}25 \times 10^{-5}$	$+1{\cdot}21 \pm 0{\cdot}66$	5×10^{-4}	$+1{\cdot}16 \pm 0{\cdot}53$
	$1{\cdot}77 \times 10^{-5}$	$+2{\cdot}08 \pm 0{\cdot}59$	$7{\cdot}07 \times 10^{-4}$	$+1{\cdot}70 \pm 0{\cdot}26$
	$2{\cdot}5 \times 10^{-5}$	$+2{\cdot}91 \pm 0{\cdot}55$	1×10^{-3}	$+2{\cdot}28 \pm 0{\cdot}38$
	$3{\cdot}54 \times 10^{-5}$	$+3{\cdot}59 \pm 0{\cdot}41$	$1{\cdot}41 \times 10^{-3}$	$+2{\cdot}73 \pm 0{\cdot}46$
	5×10^{-5}	$+4{\cdot}53 \pm 0{\cdot}56$	2×10^{-3}	$+3{\cdot}30 \pm 0{\cdot}40$
Female	$8{\cdot}8 \times 10^{-6}$	$+0{\cdot}24 \pm 0{\cdot}39$	$3{\cdot}50 \times 10^{-4}$	$+0{\cdot}48 \pm 0{\cdot}29$
	$1{\cdot}57 \times 10^{-5}$	$+0{\cdot}90 \pm 0{\cdot}38$	$6{\cdot}26 \times 10^{-4}$	$+0{\cdot}96 \pm 0{\cdot}36$
	$2{\cdot}8 \times 10^{-5}$	$+2{\cdot}03 \pm 0{\cdot}48$	$1{\cdot}12 \times 10^{-3}$	$+1{\cdot}47 \pm 0{\cdot}40$
	5×10^{-5}	$+2{\cdot}68 \pm 0{\cdot}59$	2×10^{-3}	$+2{\cdot}15 \pm 0{\cdot}49$

Table 4. *Regression equations of the results summarized in Table 3 and shown in Fig. 1, with the analyses of variance*

$Y = \Delta Q_{O_2} = Q_{O_2}$ (control) $- Q_{O_2}$ (treated tissue). $X = \ln$ [drug concentration (M) $\times 10^6$].

Source of variance	Sum of squares	Degrees of freedom	Mean squares
	Analysis of variance		
(*a*) Males: DNP. Regression equation $Y = 2{\cdot}39X - 4{\cdot}84$			
Between concentrations			
Due to regression	192·66	1	192·66
Deviation from regression	0·29	4	0·07
Within concentrations	89·83	90	1·00
Total	282·78	95	—
(*b*) Males: sodium salicylate. Regression equation $Y = 1{\cdot}53X - 8{\cdot}31$			
Between concentrations			
Due to regression	78·11	1	78·11
Deviation from regression	0·04	4	0·01
Within concentrations	52·86	90	0·59
Total	131·01	95	—
(*c*) Females: DNP. Regression equation $Y = 1{\cdot}46X - 2{\cdot}97$			
Between concentrations			
Due to regression	71·18	1	71·18
Deviation from regression	0·95	2	0·48
Within concentrations	78·62	76	1·03
Total	150·75	79	—
(*d*) Females: sodium salicylate. Regression equation $Y = 0{\cdot}95X - 5{\cdot}13$			
Between concentrations			
Due to regression	30·49	1	30·49
Deviation from regression	0·23	2	0·12
Within concentrations	53·45	76	0·70
Total	84·17	79	—

Table 5. *Combined action of salicylate and 2:4-dinitrophenol*

Results of the sequential tests of the hypotheses: that with male mouse-liver slices the mean Q_{O_2} in 1·19 mM-salicylate (S), in $2{\cdot}16 \times 10^{-5}$M-DNP (D) and in a mixture of 0·60 mM-salicylate and $1{\cdot}08 \times 10^{-5}$M-DNP (SD) were equal; the corresponding concentrations for the female were 1·07 mM-salicylate, $2{\cdot}14 \times 10^{-5}$M-DNP and 0·53 mM-salicylate plus $1{\cdot}07 \times 10^{-5}$ M-DNP. Formally, where each mean difference in Q_{O_2} was μ and its variance σ^2 the hypothesis $H_0: |\mu| < 0{\cdot}7\sigma$ was tested against $H_1: |\mu| \geqslant 0{\cdot}7\sigma$; the probability of accepting the wrong hypothesis was 0·05.

Sex	Difference tested	No. of trials	Inference	Mean difference
Male	S - D	13	Accept H_0	-0·02
	SD - S	21	Accept H_1	+0·54
	SD - D	16	Accept H_1	+0·56
Female	S - D	15	Accept H_0	-0·11
	SD - S	13	Accept H_1	+0·92
	SD - D	14	Accept H_1	+0·81

feature of the results suggests that although salicylate and DNP may have different primary actions, there is a definite relation between these actions, i.e. that the concentration–response curves of the two compounds are not independent.

The livers of male mice were more responsive to both drugs than those of females. It seems unlikely that possible variations in the metabolic rate of the female control liver slices with the oestrous cycle can account for the sex differences, for the control Q_{O_2} values were almost identical: the means for the males were −13·53, for the females −13·65, and the standard errors of these means were ±0·13 and ±0·15 respectively; these figures are derived from observations on 96 males and 80 females.

The sex differences in the response of liver slices to salicylate and DNP permit a dual comparison of the two drugs, the sexes being regarded as different preparations; furthermore, it sheds some light on the sex difference shown by mice in the hyperglycaemia due to single doses of salicylate (Sproull, 1954*a*). It had been found that although a single dose of salicylate was a powerful glycogenolytic agent in both sexes, significant hyperglycaemia was demonstrable only in the females; the present results suggest that metabolic stimulation and glycogenolysis by salicylate are not products of the same primary action. Smith, Emmens & Parkes (1947), investigating thyroid-like activity by the closed-vessel technique, found a sex difference in the response of albino mice to iodinated casein; in this case also the males were more sensitive than the females, but in contrast with the present results the slopes of the log dose–response curves were much the same, and the difference was of intercept.

The data on the combined action of salicylate and DNP eliminate the possibility of identical actions, and are also inconsistent with independence of primary actions. The occurrence of potentiation suggests a complex relation between the metabolic stimulant actions of salicylate and DNP; furthermore, especially in relation to the relatively high effective concentrations of salicylate, potentiation implies that the theory of a simple drug–receptor combination (Clark, 1937) may be inapplicable to at least one of the two drugs.

SUMMARY

1. The rate of oxygen consumption of rat-brain slices in Krebs–Ringer phosphate was increased by mM-sodium salicylate when glucose or pyruvate was the substrate, but was decreased by this concentration of salicylate with α-oxoglutarate or succinate as substrate. No appreciable effect was found at $6{\cdot}31 \times 10^{-4}$M-, $3{\cdot}98 \times 10^{-4}$M- or $2{\cdot}51 \times 10^{-4}$M-sodium salicylate when α-oxoglutarate or succinate was the substrate.

2. In glucose–Ringer a linear relation was established between the effect on oxygen consumption and the log concentration of drug over the range $6{\cdot}31 \times 10^{-4}$–1·59 mM-sodium salicylate.

3. The concentration–response curves of sodium salicylate and 2:4-dinitrophenol on the rate of oxygen consumption of mouse-liver slices were determined simultaneously over the concentration ranges $3{\cdot}5 \times 10^{-4}$–2 mM and $8{\cdot}8 \times 10^{-6}$–5×10^{-5}M respectively.

4. Linear relations between increase in Q_{O_2} and the logarithm of the drug concentration were found for both salicylate and 2:4-dinitrophenol.

5. With both drugs the gradient of the log concentration–response curve for males was significantly greater than that for females.

6. In each sex the gradient of the 2:4-dinitrophenol curve was significantly greater than that for salicylate.

7. The combined action of salicylate and 2:4-dinitrophenol on mouse-liver slices was studied, and potentiation was found.

8. It was concluded that the actions of salicylate and 2:4-dinitrophenol as metabolic stimulants *in vitro* are neither identical nor independent.

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Studies on Sulphatases

18. PREPARATION OF CHONDROITINASE-FREE CHONDROSULPHATASE FROM EXTRACTS OF *PROTEUS VULGARIS**

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Dodgson, Lloyd & Spencer (1957*a*) have shown a strain of *Proteus vulgaris* (National Collection of Type Cultures, no. 4636) to be a particularly potent source of the enzyme chondrosulphatase. The enzyme was associated with a mucopolysaccharase (chondroitinase) system which was able to degrade the polysaccharide chain of chondroitin sulphate with release of reducing substances. The method used to extract chondrosulphatase from the acetone-dried cells of *Proteus* also liberated chondroitinase into solution, and the association of the two enzymes persisted during the subsequent purification procedures. The presence of both chondrosulphatase and chondroitinase has been noted in other bacteria by several workers, including Buehler, Katzman & Doisy (1951), Reggianini (1950*a*, *b*) and Konetza, Pelczar & Burnett (1954), and in the marine mollusc *Charonia lampas* (*Triton nodiferus*), by Soda & Egami (1938).

The extent to which the activities of the two enzyme systems are interdependent (i.e. whether chondroitinase action is dependent on preliminary chondrosulphatase attack, or vice versa) is not clear, although Konetza *et al.* (1954), working with viable cells of *P. vulgaris*, obtained evidence which suggested that chondroitinase action preceded that of chondrosulphatase. With the mollusc enzymes, chondroitinase action can proceed independently of the associated sulphatase (Soda & Egami, 1938).

The present communication describes a method by which the chondrosulphatase of *P. vulgaris* can be separated from the associated chondroitinase. A preliminary account of the work has already been given (Dodgson & Lloyd, 1957).

* Part 17: Dodgson, Rose, Spencer & Thomas (1957*b*).

MATERIALS AND METHODS

Potassium chondroitin sulphate. The method of Einbinder & Schubert (1950) was modified as described by Dodgson *et al.* (1957*a*).

Testicular hyaluronidase. Both commercial (L. Light and Co., Ltd.) and laboratory (Dorfman, 1955) preparations of the enzyme were used.

Degraded chondroitin sulphate. Potassium chondroitin sulphate (50 ml. of a 0·225% solution in 0·2M-sodium acetate, adjusted to pH 5·5 with acetic acid) was incubated with testicular hyaluronidase at 37·5°, toluene being added as a preservative. A total of 150 mg. of hyaluronidase, added in three 50 mg. portions over a period of 48 hr., was necessary before exhaustion of the release of reducing substance was achieved. At the end of the incubation period the pH of the mixture was re-adjusted to 7 with N-NaOH and the preparation was kept in the frozen state until required.

Calcium phosphate gel. This was prepared by the method of Keilin & Hartree (1938); the final volume of the suspension was adjusted with water to give a concentration of 8 mg. of calcium phosphate (dry wt.)/ml.

Assay of chondrosulphatase and chondroitinase activity. The methods used were described by Dodgson *et al.* (1957*a*).

Determination of nucleic acid and protein. The nucleic acid content of the enzyme preparation at various stages during the purification procedure was determined from the ratio of the spectrophotometric readings at 280 and 260 mμ (Warburg & Christian, 1941). Protein was determined by the method of Lowry, Roseborough, Farr & Randall (1951).