The Metabolism of Sulphated Polysaccharides in Limb Atrophy in the Rat

By H. G. B. SLACK

Rheumatism Research Centre, University of Manchester

(Received 30 September 1954)

The chondroitin sulphuric acid of rib cartilage of the adult rat has been shown by administration of $\text{Na}_2^{35}\text{SO}_4$ to have a half-life of approximately 17 days (Boström, 1952*a*). It has also been shown that the uptake *in vitro* of labelled sulphate into cartilage slices involves an esterification to chondroitin sulphate and is dependent upon the integrity of some enzyme system (Boström, 1952*b*). Similarly, sulphate fixation *in vitro* has been shown to occur in different mammalian tissues and in the embryonic chick (Layton, Frenkel & Scapa, 1950; Layton, 1951*a*). This fixation appears to be inhibited by cortisone (Layton, 1951*b*), and more recently it has been found that in vitamin C-deficient guinea pigs the uptake *in vivo* of labelled sulphate into chondroitin sulphate of costal cartilage is only about one-third of the uptake in normal animals (Reddi & Nörström, 1954). Mucopolysaccharides extracted from the skeletons, pelts and viscera of rats following administration of $\text{Na}_2^{35}\text{SO}_4$ were found to have high activities in young rats and the activities decreased with increasing age (Dziewiatkowski, 1953). Mucopolysaccharides from the skin of adult rats were found to have a shorter half-life, 9–10 days, than chondroitin sulphate of rib cartilage (Boström & Gardell, 1953). The sulphur-containing fraction of these skin polysaccharides was considered by Boström & Gardell (1953) to be chondroitin sulphate but the evidence upon which this conclusion is based is incomplete. If chondroitin sulphate were in fact the only sulphur-containing polysaccharide in the skin of mammals it would mean that this particular constituent of connective tissues has entirely different rates of metabolism in different situations in the body. The faster rate of metabolism of skin polysaccharides as compared with chondroitin sulphate of costal cartilage could however be due to the occurrence in skin of sulphated polysaccharides other than chondroitin sulphate, such polysaccharides having rates of metabolism possibly quite different from that of chondroitin sulphate. Although Meyer & Chaffee (1941) believe chondroitin sulphate to be the only sulphated polysaccharide in skin, the recent work of Consden & Bird (1954) demonstrates the presence in connective tissues of other sulphated polysaccharides. Until more knowledge of the characterization of the con-

nective-tissue polysaccharides is available it would seem necessary to interpret studies of their metabolism with caution.

In a previous publication evidence was presented that collagen synthesis in the rat may continue at a normal rate in an atrophying limb (Slack, 1954). The rate of metabolic turnover of fully formed collagen in the adult rat is very slow, so that slight alterations in this rate are not easily demonstrable. In view of the reported faster rate of metabolism of the sulphated polysaccharides in connective tissue the following experiments were done to obtain information about their metabolism under conditions of limb atrophy.

METHODS

Treatment of animals

Twenty-four male albino rats with body weights varying from 280 to 400 g. were fed a normal diet throughout the experiments. The rats were divided into groups of twelve rats each. One group acted as normal controls, whilst each rat of the second group was subjected to an operation on the right hind limb, involving denervation and dislocation at the hip joint, which has previously been shown to cause massive tissue atrophy (Slack, 1954). Six weeks after the operation the rats of the second group had regained, or more than regained, their initial body weight and were free from post-operative infection.

All the animals were then given an intraperitoneal injection of 1–2 ml. of 0.9% NaCl containing $1\mu\text{C/g.}$ body weight of $\text{Na}_2^{35}\text{SO}_4$. The $\text{Na}_2^{35}\text{SO}_4$ was obtained from the Radiochemical Centre, Amersham; $1\mu\text{C}$ precipitated with $29\pm 0.3\text{ mg.}$ BaSO_4 and counted as a 1 sq.cm. disk of infinite thickness gave $2.3\pm 0.06\times 10^4$ counts/min. After receiving the injection the rats were killed by stunning at the following intervals: six normal and six operated animals at 24 hr. after injection, the remaining six normal rats at 8 days and six operated rats at 10 days after injection.

Separation of organic sulphates

Costal cartilage. Immediately after death the rib cages of the rats were cut out and the costal cartilages dissected free from muscle and surrounding connective tissue. The total costal cartilage from each rat was then finely chopped and ground with a little acid-washed fine silver sand for 5 min. The mush was transferred to a large watch glass from which the cartilaginous material could be washed off the sand into a beaker and subsequently transferred to a 50 ml. centrifuge tube. The ground cartilage was then extracted for

3 hr. at 0–5° with three changes of distilled water, being gently agitated during the extraction, and centrifuged for 10 min. at 3000 rev./min. between extractions. After the final centrifugation excess water was drained off the cartilage sediment which was then dried by washing with acetone and leaving overnight in a vacuum desiccator containing fresh anhydrous CaCl_2 . Two 100 mg. samples of the dry cartilage powder were then treated for separation of organic sulphate as described below.

Hind limbs. Both normal and atrophied hind limbs were removed from the rats by section across muscles and tendons at the level of the hip joint. All skin was removed except that around the foot. The whole limb was then chopped up with bone scissors and ground for 5 min. with a little fine acid-washed sand. The limb tissues were then separated from the sand and extracted with distilled water, and subsequently dried, as described for the costal cartilage. The mixed dry limb tissues were then divided each into two equal portions for separation of organic sulphates.

Organic sulphate extraction

Total organic sulphate. This was released as SO_4^{2-} from the dry tissue of half of each limb and from a 100 mg. portion of cartilage by boiling overnight under reflux with 100 and 50 ml., respectively, of 20% (w/v) HCl. The HCl was then removed largely by vacuum distillation on a water bath and this was completed in a vacuum desiccator over solid NaOH and P_2O_5 . The dry hydrolysates were dissolved in 30 ml. water, filtered through Whatman no. 42 paper and the sulphate precipitated in a centrifuge tube with excess BaCl_2 after addition of 5 ml. 0.05N- Na_2SO_4 as carrier sulphate. The BaSO_4 precipitation was carried out on a water bath at 85°, the pH of the redissolved hydrolysate having been adjusted to 4 with 0.1N-HCl. Precipitation at a pH much above 4 was found to give lower and variable counts on apparently identical samples from a single hydrolysate, and aggregation of the precipitate and subsequent centrifuging was aided by continuing the heating at 85° for 1 hr. After centrifuging at 2500 rev./min. for 10 min. the clear supernatant was discarded and the BaSO_4 precipitate broken up and stirred in 25 ml. water. The BaSO_4 was again centrifuged, the supernatant discarded, and resuspended in 25 ml. ethanol-ether (1:1, by vol.). The final sediment was dried in a hot-air oven at 105° for 2 hr. and stored in a vacuum desiccator overnight or until counted. BaSO_4 precipitates prepared in this way from 5 ml. of the standard 0.05N carrier Na_2SO_4 gave 29 ± 0.3 mg. dry precipitate.

Polysaccharide sulphate. Polysaccharides were separated from the other half-portions of each limb and 100 mg. portions of cartilage by digestion with papain and a subsequent purification procedure essentially as described by Einbinder & Schubert (1950). In a series of experiments preliminary to the work described in this paper, papain had been found to facilitate the separation of protein and polysaccharide under mild conditions, but the yields of polysaccharide from similar substrates varied considerably. The activating system which had been tried with the papain was thioglycolic acid in acetate buffer at pH 5, but the activating system was altered immediately the work of Kimmel & Smith (1954) became available, with marked improvement in uniformity of polysaccharide yields.

The washed tissues of each half limb were incubated for 48 hr. at 45° under xylene with 100 mg. papain (Griffin & Tatlock Ltd.) suspension, previously activated by incu-

bating at 45° for 15 min. in 250 ml. citrate buffer, pH 6, containing 0.005M cysteine and 0.005M ethylenediaminetetraacetic acid. At the end of incubation the papain digest was centrifuged at 3500 rev./min. for 15 min. The sediment was washed and centrifuged once with 100 ml. water, the original supernatant and washing combined, and reduced about one-half in volume by vacuum distillation on a water bath. The chondroitin sulphate and other polysaccharides were then purified by the method of Einbinder & Schubert (1950). The cartilage samples were treated in a similar manner except that the amount of papain used was 25 mg. Both the chondroitin sulphate obtained from the cartilage samples and the mixed polysaccharides from the limb samples gave positive metachromatic staining reactions with toluidine blue and a negative biuret reaction.

The chondroitin sulphate and mixed polysaccharide samples were hydrolysed by boiling overnight under reflux with 50 ml. 20% (w/v) HCl. After adjusting to pH 4, 5 ml. 0.05N carrier Na_2SO_4 were added and then 3 ml. 0.5M- BaCl_2 at 85° on a water bath. The BaSO_4 precipitate was then washed and dried as described above.

Residual sulphate. The residues remaining after papain digestion of the limb tissues and rib cartilage were thoroughly washed twice with distilled water, centrifuging at 3500 rev./min. for 15 min., as required. The washed residues were then hydrolysed overnight with 20% (w/v) HCl and the sulphate precipitated as BaSO_4 as described above.

Chemical analyses

Analyses of hexosamine, sulphate and nitrogen contents were determined on vacuum-dried hydrolysates of the polysaccharides.

Hexosamine. This was determined by the procedure of Elson & Morgan (1933) as modified by Schloss (1948; see Einbinder & Schubert, 1950).

Total nitrogen. This was determined by the procedure of Chibnall, Rees & Williams (1943).

Sulphate determinations. These were made by the method of Dodgson & Spencer (1953) except that ethanol used for washing the benzidine sulphate precipitates was previously saturated with benzidine sulphate and reproducible results were best obtained in the range 25–150 μg . sulphate. The method gave satisfactory reproducibility with the purified polysaccharide hydrolysates but none of the many modifications of the benzidine method for sulphates tried gave very reliable results on hydrolysates of the whole limb tissues or limb residues after papain digestion. Nor was the reproducibility of these results notably improved by precipitating with 4-amino-4'-chlorodiphenyl in place of benzidine (Jones & Letham, 1954). The estimation of small amounts of sulphate in the presence of considerable amounts of free amino acids, phosphates and chlorides presents a very difficult problem. The most consistent figures for sulphate content in these mixed-tissue hydrolysates were obtained by gravimetric determination as precipitated BaSO_4 , the precipitates being thoroughly washed in dilute HCl, water and then ethanol to reduce errors due to adsorption phenomena during precipitation. These results are shown in Table 1.

Radioactivity measurements. 28–30 mg. of the dried BaSO_4 precipitates were compressed by an accurately machined stainless-steel plunger in polythene counting pans giving 1 sq.cm. surface area and counted as 'infinitely thick' films using standard scaling equipment with a thin

Table 1. *Sulphate analyses*

Fraction I: total organic sulphate; fraction III: residual sulphate of half total mixed tissues of hind limbs and 100 mg. costal cartilage samples.

Mean of six determinations on each sample.

Source	Total sulphate			Residue sulphate		
	Mean	Max. (mg. SO_4^{2-})	Min.	Mean	Max. (mg. SO_4^{2-})	Min.
Normal half limb	3.1	3.9	2.6	0.8	1.1	0.6
Atrophied half limb	2.2	2.9	1.5	0.5	0.8	0.2
Normal cartilage (100 mg.)	2.6	2.8	2.3	0.7	0.9	0.6
Operated cartilage (100 mg.)	2.6	2.9	2.2	0.6	0.9	0.4

mica end-window Geiger-Müller tube. $1\mu\text{C Na}_2^{35}\text{SO}_4$ counted in this manner gave $2.3 \pm 0.06 \times 10^4$ counts/min., i.e. a dilution of approximately 1:100. All counts have been corrected for decay of the $\text{Na}_2^{35}\text{SO}_4$.

RESULTS

Normal rats. After removal of freely water-soluble sulphate, three fractions of bound sulphate were obtained. Fraction I, total organic sulphate, is sulphate derived from hydrolysis of one-half portions of the total mixed limb tissues in the case of the limbs and from 100 mg. samples of dry material in the case of the costal cartilage. Fraction II, polysaccharide sulphate, is the sulphate obtained from hydrolysis of 10 mg. dry samples of purified polysaccharide material obtained by papain extraction of one-half portions of the total mixed limb tissues and from 100 mg. samples of costal cartilage. Fraction III, residual sulphate, is the sulphate

obtained after hydrolysis of the washed residues remaining after papain extraction of the limb tissues and costal cartilage. In all instances the tissues were ground up and extracted initially with at least two changes each of 250 ml. distilled water. Supernatant water from a third extraction gave no appreciable precipitate with BaCl_2 , and carrier BaSO_4 precipitated from this third water extraction showed a radioactivity of only about twice the background count of 8/min. Addition of carrier Na_2SO_4 to the ground-up material before a third water extraction resulted in the removal of traces only of further radioactive sulphate. It is considered, therefore, that all these three fractions of sulphate must be entirely, or almost entirely, organically bound sulphate.

Table 2 shows the radioactivities found in these three fractions of sulphate extracted from the costal cartilage and limb tissues of normal rats. If we

Table 2. *Radioactivities of total bound sulphate, polysaccharide sulphate and residual sulphate fractions from normal rats injected with $1\mu\text{C/g. body weight Na}_2^{35}\text{SO}_4$*

Each of the three fractions was prepared from 100 mg. air-dry samples in the case of costal cartilage and from one-half of total pooled tissues of one hind limb. Sulphate in each of the hydrolysed fractions was precipitated with 29 ± 0.3 mg. carrier BaSO_4 , and the radioactivities are expressed as counts/min./sq.cm. disk of infinite thickness. All counts are corrected for radioactive decay.

Rat no.	Interval between injection and killing (days)	I. Total organic sulphate		II. Polysaccharide sulphate		III. Residual sulphate	
		Limb	Cartilage	Limb	Cartilage	Limb	Cartilage
41a	1	2678	3015	1533	2754	715	674
41b	1	2291	2837	1147	2137	402	321
41c	1	2742	3635	1080	3135	485	406
41d	1	2862	2100	1023	2052	670	529
41e	1	3040	2652	1145	2441	602	701
41f	1	2625	2989	921	2589	510	447
	Means	2706	2871	1142	2518	564	513
53	8	1294	1019	614	1522	801	420
54	8	1964	2342	753	2070	642	834
55	8	1970	2590	472	1802	944	802
56	8	1380	1306	551	1670	531	621
57	8	1143	1415	640	1576	1015	765
58	8	2032	2160	864	1906	875	655
	Means	1630	1805	649	1758	801	683
Differences (%) between means at 1 day and 8 days		-40	-37	-43	-30	+42	+33

consider first the activity/time relationships, it will be seen that there is a striking difference between the residual sulphate (fraction III) and fractions I and II. The residual sulphate from both limb and cartilage shows a gain in activity over the period 1-8 days, whereas in this time interval both the total bound sulphate and polysaccharide sulphate fractions show a loss of activity. The experimental findings seem to be sufficiently consistent to justify the conclusion that in the young adult rat some portion of the sulphate radioactivity is incorporated into a sulphate-containing tissue constituent with a slow rate of turnover. That this gain in activity with time is seen in the residual sulphate fraction could then be explained by the relative insolubility of this tissue constituent as compared with the polysaccharides extracted in fraction II, the more soluble polysaccharides with faster rates of turnover having been removed in the preceding fractionation procedures. The total bound sulphate shows a loss of 40 %, of its 24 hr. activity, at 8 days in the limb tissues and a similar loss, 37 %, in the cartilage. Since this total bound sulphate fraction includes the residual sulphate the observed loss of activity with time must be the sum of the changes in activity in at least two sulphate components, one of which must have a relatively fast rate of turnover. In the case of fraction II, the purified polysaccharide sulphate shows some difference in loss of activity with time as between limb tissues and cartilage. In the polysaccharides from limb tissues there is a loss of 43 % of its 24 hr. activity at 8 days, whereas in the costal cartilage the loss of activity at 8 days is only 30 %.

8 days is similar to the loss of activity in chondroitin sulphate from rat costal cartilage calculated from the data of Boström (1952*a*), i.e. about 27 % in 8 days. The higher rate of loss of activity with time in the polysaccharide fraction II from limb tissues, 43 %, as compared with that from costal cartilage, suggests the presence in the limb tissues of one or more sulphated polysaccharides with a higher rate of turnover than chondroitin sulphate.

The presence of several polysaccharides in the fraction II preparations could be demonstrated by ionophoresis. Using horizontal ionophoresis similar to the method of Consden & Stanier (1952), but with platinum-KCl electrodes and agar bridges, Whatman no. 1 paper in borate buffer, pH 9.4, separated the fraction II polysaccharides from limb tissue into four components. Three of these components stained with Alcian blue and showed no preferential staining with protein stains such as azocarmine. The fourth component, which migrated very slowly, stained well with azocarmine and only faintly with Alcian blue. (Although Alcian blue (Imperial Chemical Industries Ltd.) is not a specific stain for polysaccharides it has proved very useful in treating the migrating bands in paper ionophoresis since the background staining of the paper can be reduced to a faint blue by washing in 20 % (v/v) aqueous acetic acid, and this enables much smaller amounts of polysaccharide to be visualized than is the case with toluidine blue.) Under these conditions of ionophoresis the fraction II polysaccharides from costal cartilage showed three components. Two of these stained only with Alcian blue and not at all

Table 3. *Analysis of polysaccharide fraction of costal cartilage and limb tissues*

Figures are means of six determinations each on separate samples.

Source	Nitrogen (g./100 g. dry substance)	Sulphate (g./100 g. dry substance)	Hexosamine
Cartilage of normal rats	2.4	13.9	25.7
Cartilage of operated rats	2.6	13.2	23.8
Normal limb	3.4	12.2	21.2
Atrophied limb	3.8	12.6	22.7
Theoretical (K chondroitin sulphate + 4 H ₂ O; Einbinder & Schubert, 1950)	2.3	15.8	29.5

The polysaccharide extracted from the costal cartilage in this fraction, II, is presumably chondroitin sulphate, since (1) it has the same mobility on paper electrophoresis as chondroitin sulphate prepared according to Einbinder & Schubert (1950) from ox tendon, (2) it has the same staining reactions with toluidine blue and Alcian blue, and (3) the analysis of nitrogen, sulphate and hexosamine contents is in fair agreement with the published figures for chondroitin sulphate (see Table 3). Furthermore, the loss of 30 % of its activity in

with azocarmine, the faster of the two being present in much greater amount than the slower. The third component moving more slowly than the first two stained only with the azocarmine and would appear to be a protein. Chondroitin sulphate, prepared according to Einbinder & Schubert (1950), behaved similarly on ionophoresis showing one major polysaccharide component and a slower minor component. A third, even slower, faint protein component was constantly present in all preparations examined.

Operated rats. In Table 4 are shown the observed sulphate radioactivities in the operated rats. In these rats the three sulphate fractions, total organic sulphate, polysaccharide sulphate and residual sulphate, were obtained from 100 mg. costal cartilage and from half samples of both the atrophied hind limb and its opposite control limb. It will be seen that the losses of activity with time in all three

from costal cartilage are substantially greater than in the normal rats even when the figures are corrected to a time interval of 8 days. This is true also of all three sulphate fractions from the control hind limbs of these rats.

It seems, therefore, that in addition to the local process of atrophy in the operated hind limb, the effect of operation has been to produce a general,

Table 4. *Radioactivities of total bound sulphate, polysaccharide sulphate and residual sulphate fractions from rats injected with $1\mu\text{C/g. body weight Na}_2^{35}\text{SO}_4$ 6 weeks after operation to produce atrophy of one hind limb*

Each of the three fractions is derived from either 100 mg. costal cartilage or from half the total pooled tissues of the atrophied hind limb or the opposite normal hind limb. As in Table 2, radioactivities, corrected for decay, are expressed as counts/min./sq.cm. infinite thickness disk of sulphate samples precipitated with 29 ± 0.3 mg. carrier BaSO_4 .

Rat no.	Interval between injection and killing (days)	I. Total organic sulphate			II. Polysaccharide sulphate			III. Residual sulphate		
		Control limb	Atrophied limb	Cartilage	Control limb	Atrophied limb	Cartilage	Control limb	Atrophied limb	Cartilage
48	1	3893	4195	2372	740	1995	2344	615	281	876
45	1	2866	3973	4341	874	2165	2842	204	89	382
44	1	4790	5050	5261	916	2670	2892	522	178	714
43	1	4181	4982	4731	1041	3081	3410	461	142	556
42	1	3107	4603	3872	1115	2820	3200	356	171	872
41	1	1923	2892	2640	712	1629	2687	312	92	316
	Means	3460	4282	3869	900	2393	2896	412	159	623
52	10	654	876	1160	272	432	2276	276	64	432
51	10	1563	915	1862	190	241	1332	475	192	701
50	10	1018	841	1582	246	405	1440	302	161	617
49	10	1106	702	1426	381	276	2104	216	103	651
47	10	1842	1275	2091	373	435	1853	509	83	714
46	10	1715	1105	1742	346	356	1298	401	126	510
	Means	1316	952	1644	301	358	1717	363	121	604
Differences (%) between means:										
At 1 day and 10 days		-62	-78	-58	-67	-85	-41	-12	-25	-3
At 1 day and 8 days*		-50	-62	-46	-54	-68	-33	-10	-20	-2

* Calculated on the assumption that radioactivity falls linearly with time.

sulphate fractions from all the tissues are substantially greater than in the normal rats. The time interval between injection of $\text{Na}_2^{35}\text{SO}_4$ and killing in these rats is longer, 10 days instead of 8 days, and this will of course account for some of the increased losses of activity. But even if approximate correction for the time difference is made the losses of activity still remain substantially greater than in the normal animals with the single exception of the polysaccharide sulphate from costal cartilage. In this case the loss of activity at 10 days is 41 %. If the loss of activity from 8 to 10 days is assumed to be nearly linear this would give a figure of 33 % loss at 8 days as compared with 30 % in the normal rats, a difference which is not significant in a small-scale experiment. On the other hand, the losses of activity in both total sulphate and residual sulphate

probably temporary, increase in the rate of turnover of one or more tissue polysaccharides. It will be seen that the highest losses of activity with time are found in all three sulphate fractions from the atrophying limbs. In this experiment the process of limb atrophy had been proceeding some 6 weeks only and was still in the active phase. These relatively high rates of metabolism of tissue polysaccharides may not be observed when the atrophying process has been proceeding for a long time. In a small separate experiment two rats operated on 12 months previously to produce limb atrophy were injected with $1\mu\text{C/g. body weight Na}_2^{35}\text{SO}_4$. One rat was killed 48 hr. and the other 8 days after injection, and polysaccharide sulphate fraction II separated from the residual atrophied limbs and the normal control limbs. The sulphate activity in

the atrophied limb was less than one-tenth of that in the opposite control limb at 48 hr. In the rat killed at 8 days the polysaccharide sulphate activity was similar to that at 48 hr. in the atrophied limb, whereas the control limb showed a 34% lower activity. The increased rates of polysaccharide metabolism observed in the present experiment may therefore be found only in the earlier phase of tissue atrophy.

DISCUSSION

Since it is possible to demonstrate the presence in limb extracts of at least three polysaccharides, and in the costal cartilage of two polysaccharides, it seems probable that the observed losses of sulphate activity with time represent the sum of the turnover rates of two or more polysaccharides. The polysaccharide sulphate fraction II from costal cartilage appears to be most nearly a single substance on ionophoresis, the major chondroitin sulphate component being present in much larger amount than the slower moving polysaccharide. The distribution of $\text{Na}_2^{35}\text{SO}_4$ administered to the rat amongst these several polysaccharides is at present unknown, but it is hoped to obtain this information by autoradiography of ionophoresis papers and elution methods. Although only one polysaccharide may be present in relatively small amount in a mixed tissue fraction such a component may be relatively heavily labelled with isotopic sulphur and show a high rate of turnover. In this event small variations in amounts of the polysaccharide would result in marked differences in the observed activity/time curves of the total polysaccharide sulphate. Sulphated polysaccharides with a substantially faster rate of turnover than chondroitin sulphate from costal cartilage have not to date been separately identified and determined. Recently, however, this has been found to be the case for hyaluronic acid extracted from adult rat skin by Schiller, Mathews & Dorfman (1954). In experiments using [*carboxy*- ^{14}C]acetate in rats, they found a rate of turnover of approximately 2 days for hyaluronic acid but at least one sulphated substance with a much slower turnover. On the other hand, the fact that in normal rats there is a gain in activity of the residual sulphate fraction in both limb tissues and cartilage suggests the presence of a sulphated polysaccharide with a very slow rate of turnover. The fall in activity of this fraction in both limb tissues and cartilage of operated animals would be explicable assuming a generally higher rate of tissue metabolism and decreased laying down of stable components of long turnover rate. This would then account for the greater loss of activity with time in all three sulphate fractions from the atrophying limbs.

SUMMARY

1. Two groups of adult rats, twelve normal controls and twelve rats operated upon to produce atrophy of one hind limb, were injected with $1\mu\text{C/g.}$ body weight $\text{Na}_2^{35}\text{SO}_4$, and killed at two intervals of time following injection, half of each group at 24 hr. and half at 8–10 days.

2. Sulphate was recovered and counted, as BaSO_4 , from three fractions of costal cartilage and mixed limb tissues of these rats, i.e., the total bound sulphate, the polysaccharide sulphate prepared from papain extracts of the tissues, and the residual sulphate remaining in the tissues after extraction of the polysaccharide sulphate.

3. In the normal rats the polysaccharide sulphate from costal cartilage showed a loss of activity with time corresponding to a rate of turnover of about 17 days. This is in agreement with the rate of turnover of chondroitin sulphate from costal cartilage of normal adult rats found by Boström (1952*a*).

4. The polysaccharide sulphate of limb tissues of the normal rats shows a greater loss of activity with time than does the cartilage polysaccharide. Total bound sulphate of both limb tissues and cartilage, however, show similar losses, both being greater than that of cartilage polysaccharide.

5. The residual sulphate fraction from both limb tissues and cartilage actually shows a gain in activity during the 8 days of the experiment in the normal rats.

6. In the operated rats all three sulphate fractions show greater losses of activity during the 10 days of the experiment than in the normal rats with the exception of the cartilage polysaccharide fraction. The difference is particularly marked in the residual sulphate fractions which all show a loss of activity in contrast to the gain in activity in the normal rats.

7. The result of the operation appears to be an increased rate of metabolism of one or more polysaccharides in the tissues generally and in particular in the atrophying limbs.

I am indebted to Professor J. H. Kellgren for the operative technique and for constant advice and encouragement, and to Dr H. M. Muir for suggesting the use of papain.

This work was made possible by a grant from the Nuffield Foundation for research into the metabolism of extracellular substances.

REFERENCES

- Boström, H. (1952*a*). *J. biol. Chem.* **196**, 477.
Boström, H. (1952*b*). *J. biol. Chem.* **196**, 483.
Boström, H. & Gardell, S. (1953). *Acta chem. scand.* **7**, 216.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Consden, R. & Bird, R. (1954). *Nature, Lond.*, **173**, 996.
Consden, R., Glynn, L. E. & Stanier, W. M. (1953). *Biochem. J.* **55**, 248.

- Consden, R. & Stanier, W. M. (1952). *Nature, Lond.*, **169**, 783.
- Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 436.
- Dziewiatkowski, D. D. (1953). *J. expt. Med.* **98**, 119.
- Einbinder, J. & Schubert, M. (1950). *J. biol. Chem.* **185**, 725.
- Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
- Jones, A. S. & Letham, D. S. (1954). *Chem. & Ind.* **23**, 662.
- Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
- Layton, L. L. (1951a). *Cancer, Lond.*, **4**, 198.
- Layton, L. L. (1951b). *Proc. Soc. exp. Biol., N.Y.*, **6**, 596.
- Layton, L. L., Frenkel, D. R. & Scapa, S. (1950). *Cancer, Lond.*, **3**, 725.
- Meyer, K. & Chaffee, E. J. (1941). *J. biol. Chem.* **138**, 491.
- Reddi, K. K. & Nörstrom, A. (1954). *Nature, Lond.*, **173**, 1232.
- Schiller, S., Mathews, M. B. & Dorfman, A. (1954). *Fed. Proc.* **13**, 290.
- Slack, H. G. B. (1954). *Clin. Sci.* **13**, 155.

The Use of Radioactive Isotopes in Immunological Investigations

7. LABELLED PROTEINS AND PROTEIN ANTIGENS CONTAINING BOTH IODINE AND MUSTARD GAS SULPHONE GROUPS*

By G. E. FRANCIS, W. MULLIGAN† AND A. WORMALL

Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C. 1

(Received 3 December 1954)

In previous communications we have described the use of artificial protein antigens containing either iodine or mustard gas sulphone (di-2-chloroethyl sulphone; HO_2) in quantitative investigations on the precipitin reaction (Bournsell, Dewey, Francis & Wormall, 1947; Banks, Francis, Mulligan & Wormall, 1951a, b). In continuation of this work we wished to prepare antibodies to protein antigens containing two different introduced determinant groups, in order to study the reaction of these antibodies with antigens containing either or both of these groups.

Haurowitz & Schwerin (1943) have studied the properties of antibodies to iodinated azoproteins, but since, in these antigens, both determinant groups are mainly attached to tyrosine residues, and any one tyrosine residue can contain only one of these substituent groups, they are presumably well separated in the molecule. When proteins are treated with HO_2 , however, the reaction is mainly with the free amino groups, i.e. largely with the lysine residues, and the introduction of both iodine and HO_2 groups into the same protein molecule should thus yield an antigen containing two different determinant groups close together in some parts of the molecule and well separated in other parts, depending on the distribution of the tyrosine and lysine residues.

Natural protein antigens do not normally contain outstanding dominant groups comparable with those in chemically altered or conjugated protein antigens, and their serological specificity is ap-

parently related to characteristic spatial arrangements of groups of amino acids in the surface of the molecule. Thus it appears probable that their serological specificity is determined more by the configuration of a relatively large part of the molecule than by small isolated parts. Artificial antigens having different dominant groups attached to different amino acid residues might, therefore, more closely simulate the behaviour of natural protein antigens than do iodinated azoproteins.

In the experiments described here a study has been made of the conditions under which proteins can be labelled with both iodine and 'sulphone groups'. For some biological investigations it is advantageous to have two or more readily detectable labels attached to different parts of the protein molecule, with minimal chemical change in the protein, and we have, therefore, extended our experiments to include the preparation of protein derivatives containing small numbers only of iodine and sulphone groups, using reagents containing ^{131}I and ^{35}S respectively.

MATERIALS

Ovalbumin. Solutions of thrice-recrystallized hen ovalbumin in 0.9% (w/v) NaCl were used.

Serum globulins. The mixed horse serum globulins were prepared as described previously (Banks *et al.* 1951a).

Rabbit serum γ -globulins. These were prepared by the method of Kekwick (1940).

Iodine solution containing ^{131}I . Except where otherwise stated, this was prepared as described by Francis, Mulligan & Wormall (1951).

Di-2-chloroethyl sulphone containing ^{35}S . This was prepared essentially as described by Bournsell, Francis & Wormall (1946). In some preparations, however, the thio-

* Paper 6 of this series: Banks, Francis, Mulligan & Wormall (1951b).

† Present address: Department of Biochemistry, Glasgow University Veterinary School.