The Deposition of ^{35}S in Cortical Bone

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As part of the study of the site of deposition of radioactive isotopes in bone tissue, carrier-free sodium [^{35}S]sulphate was injected into rabbits and found to be concentrated in the same areas of active bone growth as is ^{90}Sr . Similar localization has been reported in the bone of rats (Dziewiatkowski, 1951, 1952*a, b*, 1954; Belanger, 1954) and of growing dogs (Lacroix, 1954; Engfeldt, Engström & Boström, 1954; Engfeldt & Hjertquist, 1954). Since [^{35}S]sulphate is known to be incorporated in high concentration in the chondroitin sulphate of the cartilage in the epiphyseal plate (Dziewiatkowski, 1951), it appeared of interest to determine whether ^{35}S was associated in bone with the apatite crystal or with an organic constituent of the matrix, or possibly with both. The problem was investigated in the following manner: (1) estimating the amounts of ^{35}S in decalcified bone and in the decalcifying solution at various times after injection; (2) extracting the substance containing ^{35}S from decalcified bone and identifying it; (3) analysing in detail the exact site of deposition of ^{35}S compared with other radioactive isotopes, and studying by autoradiography the effect of various agents that might be expected to remove sulphur compounds from bone sections.

EXPERIMENTAL

Rabbits. The rabbits used were of the same stock as those employed in previous experiments (Kidman, Tutt & Vaughan, 1950; Owen, Jowsey & Vaughan, 1955); they were fed on a diet of oats, hay and cabbage.

Estimation of the ^{35}S in decalcified bone and in the decalcifying solution. One rabbit aged 14 weeks and three rabbits aged 6 weeks were injected with three single doses of $\text{Na}_2^{35}\text{SO}_4$ (1 mc/kg.) within 8 hr. The older rabbit and one of the younger were killed 24 hr. later and the other two 7 days later. The long bones were dissected, the ends removed below the epiphyseal plates and cleared of flesh and marrow, and each shaft was decalcified in approximately 40 ml. of ethylenediaminetetraacetic acid (EDTA) solution (Srebný & Nikiforuk, 1951). This was made up by adding a 16% (w/v) NaOH solution to a 28% (w/v) solution of EDTA until the pH was 7.2, producing a mixture of the tri- and tetra-sodium salts. After decalcification, which took about 14 days, according to the size of the shaft, the matrix was dissolved in 10 ml. of concentrated HNO_3 . The radioactivity of both the fractions was estimated.

Isolation of bone-matrix substances. For the purpose of identifying the sulphur-containing compound remaining in cortical bone after decalcification two rabbits, aged 7 weeks, received three doses of $\text{Na}_2^{35}\text{SO}_4$ within 8 hr. (450 $\mu\text{C/kg.}$ in each dose). They were killed 24 hr. later. The humeri, radii, femora and tibiae were removed. The ends of the bones were sawn off below the epiphyseal plate and discarded. The marrow was removed from the shafts, which were then decalcified in a measured volume of sodium ethylenediaminetetraacetate adjusted to a pH of 7.2. Analyses were carried out on the bone matrix remaining after decalcification.

Autoradiographic experiment. One rabbit aged 6–8 weeks and one rabbit aged 3 months received three doses of $\text{Na}_2^{35}\text{SO}_4$ within 8 hr. (1 mc/kg. in each dose) and were killed 7 days later. One rabbit aged 14 weeks received the same dose and was killed 24 hr. later, and other 12-week-old rabbits were killed 9 and 21 days after injection.

Measurement of radioactivity

Solutions. The activities of solutions were measured by using a mica end-window (1 in. diameter) counter tube (General Electric Co., London, W.C. 2, Type E.H.M. 2) and 20 ml. of liquid as an 'infinitely thick' sample. The liquid was contained in a glass Petri dish (2 in. diameter) carried on the slide of a lead castle (Labgear, Cambridge) so that the distance between the surface of the liquid and the counter window was 5 mm. With the rabbits killed at 7 days after the injection the activity of the decalcifying solution was low, and, in order to increase the observed count-rates, the measurements were repeated after the total volume had been reduced to 20 ml. by evaporation. The solutions were opalescent but the solid matter remained suspended during the time of counting, and there was reasonable agreement between the measured activities of the original and concentrated samples.

Although measurement of radioactivity with liquid samples in this way is not as sensitive as with solid samples obtained by evaporating large volumes to dryness, it proves more reliable for comparing the activities of samples and standards having different contents of organic matter. The errors due to the different self-absorption in the solid samples may then be difficult to assess. The background was 20.5 ± 0.5 counts/min.

Extracted muco-substances. Counts were made of solids at infinite thickness on polythene specimen carriers (2 sq.cm.), except where otherwise stated.

Paper chromatography. Chromatograms were prepared on Whatman no. 1 paper by descending elution with the organic phase of a *n*-butanol-ethanol-water (4:1:5, v/v) mixture. The positions of amino acids were revealed by spraying the chromatograms with 1% ninhydrin in

butanol. Reducing sugars were detected by means of aniline hydrogen phthalate (Partridge, 1949).

Paper electrophoresis. The apparatus described by Cremer & Tiselius (1950) was employed, with chlorobenzene as a cooling agent. All examinations were carried out in 0.07M barbiturate buffer at pH 8.8, on Whatman no. 1 paper. The applied starting potential was 8.4v/cm. Sulphated substances were located by staining with toluidine blue (0.075% in 50% v/v aqueous ethanol) and removing the background colour with 50% (v/v) ethanol (Pasternak & Kent, 1951).

Histological technique. Histological sections stained by the periodic acid-Schiff reagent (PAS) (Pearse, 1953), toluidine blue and eosinazure were prepared as described by Jowsey, Sissons & Vaughan (in preparation).

Microradiographs and autoradiographs were prepared from thin cross-sections of bone from the upper half of the tibia which had been embedded in Perspex (Jowsey, Owen & Vaughan, 1953; Owen *et al.* 1955; Jowsey, 1955). In some experiments fast experimental film (Kodak Scientific Experimental Plate No. V1001) was used; in others high-resolution film (Kodak M.R. autoradiographic stripping film) was used. Fast experimental film was fixed in 'hypo' for 2.5 min. at 18° instead of the usual 5 min. at 18° and washed well to minimize effect of fixation on the sections. The localization of the isotope was noted and the film floated off the sections and mounted separately. A few representative sections were kept with the film on as controls, so that any differences between these and the treated sections could be checked at the end of the experiment. The sections were immersed in CHCl_3 for 3 hr. to remove any traces of Perspex and to allow the solutions to penetrate the tissues. They were hydrated and washed in distilled water, and each section was demineralized under one of the following conditions: (a) EDTA for 2 hr.; (b) EDTA for 15 hr.; (c) 0.5% HNO_3 (v/v) for 0.5 hr.; (d) 0.5% HCl (w/v) for 0.5 hr. The decalcified sections were then remounted a second time and autoradiographs prepared from them. The second autoradiographs were left on the section and compared with the first. Correction was made for the decay of ^{35}S in calculating the exposure time for the second autoradiographs.

Some sections, untreated with the decalcifying agents, were remounted to allow a second autoradiograph to be made in order to ensure that the developer and fixer had no effect on the sections in the first autoradiographic preparation. Other sections were stained by the periodic acid-Schiff technique before a second autoradiograph was prepared, in order to determine whether there was any connexion between PAS-positive substances and the deposition of sulphur.

Grain counts. The reduction of the silver grains of photographic film by radiations from radioactive isotopes in tissues in contact with the film forms the basis of all autoradiographic techniques (Doniach & Pelc, 1950; Bourne, 1952). The number of developed grains in the autoradiograph is proportional to the activity present in the tissue. A comparison of the number of grains in different parts of the tissue may be used as an indication of the amount of radioactive isotope present. Many modifications of this principle are in use (Doniach & Pelc, 1950; Lajtha, 1954). In the present instance grain counts were made by means of an oil-immersion objective and eye-piece ($\times 10$ magnification) containing a small disk with 0.5 mm. aperture. Three

counts were made over an area of intense localization and an average was taken. The numbers of grains in areas of less intense uptake were determined in the same way and expressed as percentages of the counts in areas of intense localization, the figure for counts over film away from the section being subtracted in both cases. This method, in which relatively thick sections rather than single cells are used, has severe limitations, since each autoradiograph may differ from another because of variations in (i) section thickness, (ii) the distance between the section and the film, (iii) the precise conditions of development and (iv) exposure time. Counts made on different parts of an autoradiograph prepared from a single section are more comparable than counts made on autoradiographs prepared from different sections. If every effort is made to keep conditions constant such counts may be used to give qualitative results.

RESULTS

Calculation of the amounts of ^{35}S in decalcified bone, in whole bone and in decalcifying solution (EDTA). The amount of ^{35}S in decalcified bone, i.e. in the nitric acid fraction, has been expressed as a percentage of the total ^{35}S in each bone, the limits being those given by the standard deviations in values of activity for both the EDTA and the dissolved matrix. By comparison with a ^{35}S standard solution of known activity the total activity in each bone has also been calculated as a percentage of the total dose injected. These figures are shown in Table 1.

The amount found in the EDTA is the difference between that found in the whole bone and in the acid fraction.

The amount of ^{35}S retained is small, and, even for these large doses, the count-rates above background which were obtained with this technique of measurement were of the order of the background count-rate itself; consequently the statistical accuracy is not high, the standard deviation for some of the results being greater than $\pm 10\%$. However, it appears to be established for all bones that the proportion of the total ^{35}S in the decalcifying solution is greater at 24 hr. than at 7 days. Also, by comparing the three rabbits which were of similar age, it appears that the total amount of ^{35}S present in each bone is much lower at 7 days than at 24 hr., only about 15% of the activity at 24 hr. remaining in the femora and about 25% in the other bones.

Extraction of sulphated muco-substances from decalcified bone. The decalcified bones (4.9 g. moist weight) were shaken at room temperature for 10 hr. with 2% (w/v) aqueous potassium hydroxide (50 ml.). The extract was removed and the bones were re-extracted by the same procedure. The combined extracts, after centrifuging, were deproteinized by shaking ten times with a 4:1 (v/v) mixture of chloroform and pentanol for 1 hr. each

time (Sevag, 1934). The final aqueous solution failed to yield a precipitate when treated with glacial acetic acid. When ethanol (3 vol.) was added, a solid was obtained which was removed and dried with absolute ethanol and ether (yield 0.119 g., 30 counts/min.).

Paper-electrophoretic separation of the radioactive product. A sample (1 mg.) in barbiturate buffer (pH 8.8) was submitted to paper-electrophoretic examination. Non-radioactive chondroitin sulphate from bovine trachea (Bray, Gregory & Stacey, 1944) and a mixture of this with the radioactive product were examined simultaneously. After 6 hr. the substances were located by treatment with toluidine blue. All three substances had migrated a distance of 10–15 cm., and the region of metachromatic staining and radioactivity, measured by cutting the stained paper into 2 cm. transverse strips and taking the radioactivity on each strip, were coincident in the rabbit-bone muco-substance alone and in the mixture with carrier chondroitin sulphate.

above background, which was 12.4 counts/min. ± 0.7 s.d. Even after prolonged dialysis (70 hr.) no trace of $^{35}\text{SO}_4^{2-}$ was found to have diffused through the membrane.

The solution within the membrane was found to be radioactive; this contained solid having a count of 22/min. above background. The radioactive material was not precipitated by addition of BaCl_2 (with or without carrier sulphate).

Hydrolysis of radioactive rabbit-bone product.

(a) The above dialysed product (1 ml. $\equiv$ 1 mg.) was heated with 0.25 ml. of concentrated hydrochloric acid at 105° for 12 hr. Addition of 0.1N- H_2SO_4 (0.5 ml.) and 0.1N- BaCl_2 (1 ml.) resulted in the precipitation of radioactive BaSO_4 . The total precipitated activity was equivalent to 21 counts/min. ± 2.1 s.d. above background (12.4 ± 0.7 s.d.). The experiment was carried out in duplicate, 96% of the total activity of the dialysed muco-substance being recovered as $^{35}\text{SO}_4^{2-}$.

(b) Hydrolysis of 4 mg. of the solid undialysed rabbit product with 0.5 ml. of 5N-HCl at 105° for

Table 1. Proportions of ^{35}S in acid solutions of bone matrix and total bone content of ^{35}S (expressed as percentage of dose injected)

Each rabbit received 3 mc/kg. of $\text{Na}_2^{35}\text{SO}_4$.

Age of rabbit (weeks)	Time between injection and killing	Bones	^{35}S in nitric acid solution (percentage of total bone content) $\pm$ s.d.	Bone content of ^{35}S (percentage of injected dose) $\pm$ s.d.
14	24 hr.	Femora	29.5 $\pm$ 1	0.135
		Humeri	21.5 $\pm$ 1.5	0.075
6	24 hr.	Femora	18.5 $\pm$ 1.5	0.13 $\pm$ 0.005
		Humeri	9.5 $\pm$ 3.5	0.04 $\pm$ 0.005
		Tibiae	19.5 $\pm$ 4	0.05 $\pm$ 0.005
6	7 days	Femora	43 $\pm$ 6	0.020 $\pm$ 0.002
		Humeri	35 $\pm$ 10	0.008 $\pm$ 0.001
		Tibiae	36 $\pm$ 6	0.013 $\pm$ 0.001
6	7 days	Femora	43 $\pm$ 5	0.015 $\pm$ 0.001
		Humeri	53 $\pm$ 7	0.010 $\pm$ 0.0015
		Tibiae	46 $\pm$ 6	0.015 $\pm$ 0.0015

The same results were obtained when the specimens were mixed with sodium sulphate solution before application to the paper. Electrophoresis under identical conditions of a mixture of non-radioactive chondroitin sulphate and $\text{Na}_2^{35}\text{SO}_4$ gave only a non-radioactive metachromatic zone.

Dilution dialysis. A sample (4 mg.) of the radioactive rabbit-bone product was dissolved in a mixture of 4.5 ml. of water and 0.5 ml. of 1% (w/v) sodium sulphate solution. The solution was dialysed (cellophan) against distilled water (400 ml.) for 17 hr. To 10 ml. of the external liquid 0.1N sulphuric acid (1 ml.) and 0.1N barium chloride (2 ml.) were added and the resulting barium sulphate precipitate was filtered off and dried with ethanol. This precipitate showed no radioactivity

12 hr., followed by paper-chromatographic analysis by the method of Stoffyn & Jeanloz (1954), gave evidence of the presence of galactosamine.

Localization of ^{35}S as determined by autoradiography

Precise site of deposition of ^{35}S . Autoradiographs prepared from bone sections of animals in both age groups showed that ^{35}S is incorporated in areas of active bone growth in high concentration, as is ^{90}Sr (Jowsey *et al.* 1953), and that the apparent movement of the radioactive isotope with time is dependent on the apposition of new bone containing no radioactive isotope.

^{35}S is found in the cartilage cells of the epiphyseal plate. Below this the trabeculae of the primary and secondary spongiosa show concentration of

the radioactive isotope. In the primary spongiosa the deposition appears in narrow bands of bone that are laid down on the cartilage scaffolding. Grain counts suggest that this uptake is approximately half as strong as that in the cartilage of the plate. In the animal killed 21 days after injection, it is found that the cartilage has grown down from the plate, and now lies in the centre of the trabeculae which shows deposition, and that this is covered by new bone formed since injection. The ^{35}S taken up by the secondary spongiosa shows a patchy and somewhat less intense localization in bone which appears incompletely calcified in the microradiographs.

In the metaphysis and diaphysis there is deposition of ^{35}S endosteally and periosteally in the newly formed bone (Owen *et al.* 1955) and in bone at active building sites. In animals killed 7 and 21 days after injection, the endosteal and periosteal bone and the lamellae in active building sites containing ^{35}S have been overlaid by new bone formed since injection, which contains no radioactive isotope (Figs. 1 and 2).

other hand, is taken up in all the bone of low calcification, with a gradual falling-off of the intensity of the reaction as the bone becomes more completely calcified.

Those areas of growing bone which take up ^{35}S are stained metachromatically and give a positive periodic acid-Schiff reaction. In bone tissue this positive reaction may indicate a low degree of sulphation or of polymerization of matrix constituents, or a high concentration of a substance such as chondroitin sulphate. The association of high concentrations of ^{35}S in areas showing a positive periodic acid-Schiff reaction is particularly noticeable in the building sites in cortical bone. A high percentage of those sites that show a positive PAS reaction also contain ^{35}S . Not all areas showing a positive PAS, however, take up ^{35}S , since they may be surfaces where resorption is active (Heller-Steinberg, 1951) or areas which have become PAS-positive since injection.

In addition to this concentrated reaction there is a diffuse background reaction throughout bone. This background reaction is greatest in autoradiographs prepared from the bone of the rabbit killed

Fig. 1. Radioautograph of unstained cross-section of rabbit tibia (7 days after administration of $^{35}\text{SO}_4^{2-}$), showing layer of high concentration of isotope in endosteal bone overlaid with new bone showing no ^{35}S . ($\times 120$.)

Fig. 2. Radioautograph of unstained cross-section of rabbit tibia (7 days after administration of $^{35}\text{SO}_4^{2-}$), showing region of high isotopic concentration of ^{35}S over adjacent lamellae at a distance from Haversian canals. ($\times 300$.)

All the areas of localization of radioactive sulphur in the animals killed up to 9 days after injection appear incompletely calcified in the microradiographs, and other experiments (Jowsey *et al.* 1953; Owen *et al.* 1955) have shown that ^{90}Sr is deposited characteristically in the same regions. However, ^{35}S is found in a few lamellae only; ^{90}Sr , on the

24 hr. after injection, and is much reduced in animals killed 7 and 21 days after injection. At the later time-intervals grain counts suggest that the reaction is only 10–15% as strong as the earlier concentrated reaction. In the older animals there is non-Haversian bone present endosteally and periosteally, and such bone shows a background

reaction only half as intense as the reaction over Haversian bone in the same sections (Fig. 3).

Effect of demineralization on the site of deposition of ^{35}S . Grain counts in both the diffuse background and over areas of concentration on autoradiographs prepared from undecalcified sections of bone from rabbits killed 24 hr. after injection are higher than those obtained on autoradiographs prepared after decalcification by any of the methods used. The reduction is, however, much greater in the diffuse grain distribution than in areas of concentration. Grain counts on autoradiographs prepared from sections of bone from rabbits killed 7 days after injection appeared to be unaffected by decalcification.

ever, many sources of error in comparative grain counts on autoradiographs from sections treated in different ways, and until further investigations of the validity of the method have been carried out too much stress should not be laid on the quantitative results obtained. It can be shown, however, from the autoradiographs that there is considerable loss of the diffusely distributed ^{35}S with time, while the concentration of ^{35}S in new bone remains at least qualitatively high. The nature of the soluble sulphate is unknown and is to be further investigated.

The radioactive material in the decalcified bone is extractable under alkaline conditions, and in fractionation the activity becomes concentrated in

Fig. 3. Radioautograph of unstained cross-section of rabbit tibia (21 days after administration of $^{35}\text{SO}_4^{2-}$), showing distribution of ^{35}S over Haversian bone and less dense background over non-Haversian bone. *Note.* Grains of emulsion in focus; section therefore slightly out of focus; Haversian canals easily distinguished in Haversian bone, and cement lines of lamellae in non-Haversian bone. ($\times 60$.)

DISCUSSION

The observations recorded here on the effect of decalcification on the ^{35}S content of rabbit bone, as estimated by measuring the relative radioactivity of the decalcifying solution and the decalcified bone, suggest that ^{35}S is present in the bone in two forms—one that is readily removed by decalcification and one that is not. The proportion of each present varies with time after injection, thus confirming the observations of other workers (Engfeldt *et al.* 1954; Engfeldt & Hjertquist, 1954).

The autoradiographic experiment suggests that 24 hr. after injection the labile form of ^{35}S is diffuse in distribution over the bone surface, while the ^{35}S that is bound is present in the areas of active bone growth. At longer periods after injection, radiochemical and autoradiographic evidence is not in such close agreement. ^{35}S was still present, though in reduced amounts, in the decalcifying solution, but grain counts on autoradiographs prepared from sections before and after decalcification suggested no loss of ^{35}S . There are, how-

the carbohydrate fraction as opposed to the collagen and protein fractions. Though the radioactive constituent has not yet been isolated in a pure form, the following evidence suggests strongly that chondroitin sulphate or a nearly related substance is present: (a) In paper-electrophoretic experiments the substance has the same mobility as authentic non-radioactive chondroitin sulphate, alone or in dilution with non-radioactive material. (b) The isolated substance is metachromatic in the same way as other sulphated muco-substances. (c) The sole metachromatic component in paper electrophoresis is coincident with the sole radioactive component. (d) A reducing amino sugar and $^{35}\text{SO}_4^{2-}$ are detectable only after acidic hydrolysis of the isolated radioactive substance.

The radioactivity of the bone chondroitin sulphate is accounted for in terms of esterified sulphate. Dialysis of the radioactive product with added sodium sulphate results in no release of $^{35}\text{SO}_4^{2-}$, as would be expected if it was held by ionic or other readily exchangeable linkages. The radioactivity is recovered fully as $^{35}\text{SO}_4^{2-}$ by acidic hydrolysis of the muco-substance.

It is known (Florey, 1955; Kent, Whitehouse, Jennings & Florey, 1956) that duodenal mucins obtained from rabbit fistulae show a high level of radioactivity almost immediately after the intravenous administration of $\text{Na}_2^{35}\text{SO}_4$. The immediate response is due to the presence in the mucous secretion of $^{35}\text{SO}_4^{2-}$, and the amount of ester sulphate incorporated into the muco-substances rises to a maximum only 4 hr. after injection of the isotope. In this case, the presence of ionic sulphate in the mucin indicates only its availability to the mucous glands, and its retention and slow rate of incorporation suggest the existence of some intermediate biochemical 'trapping' or storage process. By analogy, the presence of ionic sulphate in bone, as generally in other tissues, is not unexpected.

The determination of the precise site of deposition of ^{35}S that is made possible by the high resolution of the present autoradiographs shows that this deposition differs from that of calcium and strontium in one important particular: calcium and strontium are distributed over the area of low calcification in an active building site, whereas sulphur lies over a few lamellae only. The strongly positive PAS reaction also occurs, not over the whole area of an active building site, but at the periphery over a few lamellae, presumably in the area where the matrix is being formed; this suggests that incorporation of the ^{35}S occurs only in newly formed matrix, whereas calcification of matrix is a continuous process occurring over a larger area.

These results are consistent with the hypothesis that two main series of biochemical processes operate during bone formation. The first series is the synthesis of a sulphated organic matrix, which the present experiments suggest is chondroitin sulphate or a closely allied substance (Rogers, 1949). The incorporation of ^{35}S into such a substance in bone points to the existence of some form of sulphate-free (or hence more PAS-positive) precursor. Such a possible precursor of chondroitin sulphate has been demonstrated in bovine cornea (Davidson & Meyer, 1954) and may have a widespread distribution. The high localization of ^{35}S at sites of sulphate esterification is therefore not unreasonable. The second series of biochemical processes involves the deposition of inorganic cations about the ionic environment provided by sulphated muco-substances. The high concentration of ^{90}Sr in areas of low calcification may be due to the fact that these areas are zones of high biochemical activity.

In addition to these synthetic processes, there is imposed the dynamic biochemical state in which a general, and in this case slow, turnover (Kent & Whitehouse, 1955) of tissue constituents occurs. A degree of redistribution (not necessarily uniform

throughout the tissue) of the isotopic material is therefore to be expected, though this may give rise to a substantially lower but more general degree of incorporation of ^{35}S , as shown by the background reaction in the autoradiographs.

SUMMARY

1. Observations on the deposition of ^{35}S in the tibiae of rabbits obtained by the use of both chemical and autoradiographic techniques are described.

2. ^{35}S is incorporated into bone in two forms: (1) a labile form throughout the bone which is in large part removed by decalcifying agents; (2) a non-labile form present in high concentration in areas of active bone growth, which is not removed by decalcifying agents.

3. The radioactive material extracted from decalcified bone has been shown to be chondroitin [^{35}S]sulphate or a closely allied substance.

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REFERENCES

- Belanger, L. F. (1954). *Canad. J. Biochem. Physiol.* **32**, 161.
- Bourne, G. H. (1952). *Biol. Rev.* **27**, 108.
- Bray, H. G., Gregory, J. E. & Stacey, M. (1944). *Biochem. J.* **38**, 142.
- Cremier, H. D. & Tiselius, A. (1950). *Biochem. Z.* **320**, 273.
- Davidson, E. A. & Meyer, K. (1954). *J. biol. Chem.* **211**, 605.
- Doniach, I. & Pelc, S. R. (1950). *Brit. J. Radiol.*, N.S., **23**, 184.
- Dziewiatkowski, D. D. (1951). *J. exp. Med.* **93**, 451.
- Dziewiatkowski, D. D. (1952a). *J. exp. Med.* **95**, 489.
- Dziewiatkowski, D. D. (1952b). *Metabolic Interrelations Trans., 4th Conf. Josiah Macy, Jr. Found., New York*.
- Dziewiatkowski, D. D. (1954). *J. exp. Med.* **99**, 283.
- Engfeldt, B., Engström, A. & Boström, H. (1954). *Exp. Cell Res.* **6**, 251.
- Engfeldt, B. & Hjertquist, S. O. (1954). *Acta path. microbiol. scand.* **35**, 205.
- Florey, H. W. (1955). *Proc. Roy. Soc. B*, **143**, 147.
- Heller-Steinberg, M. (1951). *Amer. J. Anat.* **89**, 347.
- Jowsey, J. (1955). *J. sci. Instrum.* **32**, 159.
- Jowsey, J., Owen, M. & Vaughan, J. (1953). *Brit. J. exp. Path.* **34**, 661.
- Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of the Amino Sugars*. London: Butterworths.
- Kent, P. W., Whitehouse, M. W., Jennings, M. A. & Florey, H. W. (1956). *Quart. J. exp. Physiol.* (in the Press.)
- Kidman, B., Tutt, M. L. & Vaughan, J. M. (1950). *J. Path. Bact.* **64**, 453.

- Lacroix, M. P. (1954). *Radioisotope Conf.* vol. 1, p. 134. London: Butterworths.
- Lajtha, L. G. (1954). *J. photogr. Sci.* 2, 130.
- Owen, M., Jowsey, J. & Vaughan, J. (1955). *J. Bone Jt. Surg.* 37B, 324.
- Partridge, S. M. (1949). *Nature, Lond.*, 164, 443.
- Pasternak, C. A. & Kent, P. W. (1951). *Research, Lond.*, 5, 486.
- Pearse, A. G. E. (1953). *Histochemistry Theoretical and Applied*. London: J. and A. Churchill, Ltd.
- Rogers, H. J. (1949). *Nature, Lond.*, 164, 625.
- Sevag, M. (1934). *Biochem. Z.* 273, 419.
- Srebnny, L. M. & Nikiforuk, G. (1951). *Science*, 113, 560.
- Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* 52, 373.

The Production of Polyacetylenic Compounds by Basidiomycetes: Glucose Conversion

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Polyacetylenic compounds, i.e. compounds containing two or more triple bonds in conjugation, are produced by a variety of (mainly higher) fungi when grown on synthetic or semi-synthetic media. They occur sufficiently frequently (Anchel, 1954, and unpublished observations) to make it apparent that the production of such compounds, though unusual, is not a rare freak of metabolism. The fungal polyacetylenes belong to a class of compounds which includes the complex antibiotics and an enormous variety of other 'natural products', compounds which play no obvious part in the primary metabolic processes of organisms. Despite the great practical and theoretical interest of such 'secondary metabolites', their relationship to the better-known processes, such as glycolysis or protein synthesis, is very little understood. As relatively simple members of this class of compound, and also as containing chemical units of peculiar thermodynamic instability, the fungal polyacetylenes are of especial biochemical interest. A comparison of the structures of the small number of compounds which have been fully investigated does not yet permit any significant conclusions to be drawn, and it may be noted that chains of both odd and even numbers of carbon atoms are represented.

In order to provide material for chemical studies, we have grown polyacetylene-producing fungi under a variety of conditions. The polyacetylenes have distinctive ultraviolet absorption spectra, and this has made possible the study of the course of polyacetylene production in different cultures in some detail. The present communication records observations made with an unidentified Basidiomycete, B. 841, originally isolated from white cedar by Dr Baxter at the University of Michigan. Cultures of this fungus produce hydrocyanic acid (Robbins, Rolnick & Kavanagh, 1950)

and a mixture of four closely related polyacetylenic antibiotics. The structures of the latter are indicated in Fig. 1, and details of their separation are described elsewhere (Bu'Lock, Jones & Leeming, 1955); it is sufficient to note here that all share a distinctive ultraviolet absorption spectrum (Fig. 2) by measurement of which their combined concentrations may be measured.

Fig. 1. Antibiotics isolated from the fungus B. 841 (Bu'Lock *et al.* 1955).

Fig. 2. Ultraviolet absorption spectrum of nemotinic acid. The spectra of the other polyacetylenes from the fungus B. 841, and of mixtures, differ only in detail.