Table 3. *Sedimentation constants of the extract peptide*

Concentration, %	2.00	1.50	1.00
S_{20} (Svedbergs)	0.93	1.01	1.13

Electrophoresis. The material was examined under precisely the same conditions as the exudate peptide and practically identical behaviour was observed. Electrophoretically it showed only a single component with a mobility, calculated for the cathode limb, of 0.42×10^{-4} cm.²/sec./v in close agreement with that found for the exudate peptide.

Sedimentation. Here also the results show a close parallel with those obtained for the exudate peptide. The corrected S_{20} values are given in Table 3, and the extrapolated value of $S_{20}(c \rightarrow 0) = 1.32$. At the same concentration, the rate of spread of the sedimenting boundary appeared to be slightly less than in the case of the exudate peptide.

Diffusion. An anomaly was observed here in the form of a sharp peak surmounting the main concentration gradient curve and displaced slightly to the buffer side of the main peak. The picture suggests the presence of a small percentage of a high molecular contaminant, though there is no evidence of this in the ultracentrifuge diagrams. The presence of this contaminant makes the analysis of the diffusion curves difficult. The main part of the sample, however, diffuses at a rate very near to that found for the exudate peptide. Without further purification it is doubtful whether a more detailed examination of the extract peptide sample is justified.

DISCUSSION

As seen in the main part of this paper, the crude non-dialysable fraction of the exudate yielded 96 % of a 2.5 % trichloroacetic acid soluble fraction, this

constituting the sample examined here, whereas the crude extract dialysate gave only a 20 % yield when similarly treated. It appears that the germinating spores allow only material of relatively low molecular weight, the exudate peptide, to pass into solution. The extract peptide, on the other hand, has been separated by a single fractionation from the entire spore contents and it is perhaps not surprising that it does not reach the same standard of homogeneity and monodispersity as the exudate peptide.

Evidence of some polydispersity has been adduced in both samples by viscosity measurements on fractions separated by precipitation at various ethanol concentrations. The differences were much more pronounced in the extract than in the exudate peptide, thus supporting the conclusions already reached.

SUMMARY

1. The exudate peptide from germinating spores of *B. megatherium* appears to be homogeneous in electrophoresis and in sedimentation at pH 4.5.

2. At 20°, it has a sedimentation constant at zero concentration of 1.28×10^{-13} , a diffusion coefficient of 5.88×10^{-7} and a partial specific volume of 0.655. These data give a figure of 15 300 for the molecular weight and 2.4 for the frictional ratio, pointing to marked molecular asymmetry.

3. The material is essentially monodisperse.

4. A peptide extracted from resting spores of *B. megatherium* appears to be physico-chemically identical to the exudate peptide except for the presence of a small amount of contaminant.

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The Biological Action of Substances Related to Thyroxine

9. THE EFFECT OF BUTYL 4-HYDROXY-3,5-DIODOBENZOATE ON THE DEIODINATION OF TRIIODOTHYRONINE IN THE RAT

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The metabolic interactions of butyl 4-hydroxy-3,5-diiodobenzoate (BHDB) with thyroxine and triiodothyronine in intact rats and mice have been previously reported (Sheahan, Wilkinson & MacLagan, 1951; MacLagan, Sprott & Wilkinson, 1952; MacLagan & Wilkinson, 1952; Sprott, Wilkinson & MacLagan, 1953). These studies have led us to advance the hypothesis that BHDB exerts its

effects by inhibiting the deiodination both of thyroxine and of triiodothyronine *in vivo*. Evidence in support of the first part of this theory has been obtained by studying the effect of BHDB on the urinary iodide of rats treated with thyroxine labelled in the 3' and 5' positions with ¹³¹I (Wilkinson & MacLagan, 1953a; Wilkinson, Sprott, Bowden & MacLagan, 1954). The present communication

reports a similar study with BHDB and triiodothyronine, the latter labelled with ^{131}I in the 3' position.

Preliminary reports of some of the results described in this paper have appeared elsewhere (Wilkinson & MacLagan, 1953*b*, 1954).

EXPERIMENTAL

Preparation of ^{131}I -labelled 3:5:3'-triiodo-L-thyronine

Various methods for the preparation of the labelled triiodothyronine were studied. At the suggestion of Professor A. Wormall, the isotope-exchange method (Miller, Anderson, Madison & Salley, 1944; Frieden, Lipsett & Winzler, 1948) was tried, but, though good yields of material of high specific activity were obtained, it was decided not to use this product since we could not be certain that exchange had taken place exclusively in the 3' position. A method based upon that used by Clayton, Free, Page, Somers & Woollett (1950) for the preparation of labelled thyroxine was finally employed, the product being purified via the hydrochloride by a procedure similar to that used by Gross & Pitt-Rivers (1953).

Isotope-exchange method. Of the several procedures studied the following gave the best results. Triiodothyronine (10 mg.) was suspended in a mixture of 0.1*N* acetate buffer of pH 5.3 (5 ml.) and *n*-butanol (1 ml.) containing Na^{131}I (50 μC). The mixture was shaken at intervals while being heated under reflux on a steam bath for 42 hr. The butanol was removed by evaporation at 20°/1 mm. and the residue diluted to approx. 8 ml. The solid was collected and washed with water until no radioactivity could be demonstrated in the washings (about 10 of 3–4 ml. each were required). The product was then dried over silica gel at 20°. After making allowance for radioactive decay, the total utilization was 90%. The product was shown by paper chromatography to have undergone no decomposition. When the butanol was omitted, the utilization was only 65% after similar treatment. The uptake was negligible when the mixture was shaken mechanically for 42 hr. at 20°, irrespective of the presence or absence of the solvent.

Iodination of 3:5-diiodo-L-thyronine. A solution of KI (9.6 mg., 0.058 m-equiv.) and Na^{131}I (5 mc) in water (2 ml.) was acidified with 2*N*-HCl (0.15 ml.) and covered with ether (5 ml.). The mixture was treated with 0.1*N*- H_2O_2 (1.3 ml., 0.13 m-equiv.), added over 20 min., and shaken at intervals for 4 hr. The ether layer was then transferred slowly to a solution of 3:5-diiodo-L-thyronine (15.3 mg., 0.029 m-equiv.) in 50% aqueous ethylamine (0.5 ml.). The iodination required about 2 hr., towards the end of which, water (2 ml.) was also added. The ether was removed in a current of air, and the mixture evaporated to approx. 0.5 ml. at 30°/1 mm. to remove excess ethylamine. The residue was transferred to a centrifuge tube, about 5 ml. of water being added, and the solution heated to 60°. The pH was adjusted to 4–5 with acetic acid to precipitate the product. After several hours at 0–5°, the solid was collected and washed 3 times with water (10 ml.). The crude product was dissolved in 2*N*-HCl (5 ml.) at 70–80°, the solution was filtered, and the filtrate, while still hot, adjusted to pH 4–5 with NaOH and acetic acid. It was then stored at 0–5° for 2 hr., after which the

crystalline deposit was separated in a centrifuge and washed 3 times with water (10 ml.). The product was again dissolved in 2*N*-HCl and the crystallization procedure repeated.

The 3:5:3'-triiodo-L-thyronine thereby obtained in 80% yield was shown by paper chromatography to be free from thyroxine and iodide. The chromatograms were developed by the ceric sulphate–arsenious acid method of Bowden & MacLagan (1954) and also by autoradiography on Ilford Industrial B X-ray film. The only possible contaminant not detectable by this procedure is diiodothyronine. The extent of contamination with this substance must have been small since the product had m.p. 231–232° (decomp.). Gross & Pitt-Rivers (1953) report 233–234° (decomp.). As diiodothyronine has no known physiological activity and could not be radioactive, the product was considered suitable for use in the experiments described in this paper. It had a specific activity of 61.5 $\mu\text{C}/\text{mg.}$, which after allowing for radioactive decay, corresponds to 44% of the theoretical utilization of radio-iodide.

For use in the animal experiments, a solution of 100 $\mu\text{g.}/\text{ml.}$ in 50% (v/v) aqueous propylene glycol was prepared, the pH being adjusted to 8–9 with diethanolamine. Immediately before using this solution paper chromatograms were run to ensure the absence of interfering substances. For administration to rats, the concentrated solution was diluted with physiological NaCl solution to produce the required dose in 1 ml.

Solutions of radioactive triiodothyronine appeared to be much more stable than those of labelled thyroxine (Wilkinson *et al.* 1954), for after storage for several weeks no trace of iodide could be detected chromatographically, using either the ceric sulphate–arsenious acid method or autoradiography.

Animal experiments

In each experiment male rats, each within ± 15 g. of the mean weight, which in different experiments varied from 140 to 200 g., were fed on cubed stock diet (Thomson, 1936) and tap water, both *ad lib*. Groups of three similarly treated animals were kept in metabolism cages, from which the urine and faeces were collected daily. After suitable treatment (Wilkinson *et al.* 1954) the radioactivity of the excreta was determined by counting in a liquid counter (Veall, 1948) connected to a standard scaler.

The solution of triiodothyronine was administered by subcutaneous injection. Suspensions of BHDB (50 mg./ml.) in 1% aqueous methoxycarbonylcellulose were given orally, the controls receiving only the medium by the same route.

RESULTS

The results obtained in a typical experiment in which doses of 6 $\mu\text{g.}/\text{rat}$ of triiodothyronine were given are shown in Fig. 1. As in earlier experiments half of the rats received BHDB (50 mg./rat) on the day before the triiodothyronine injection and 25 mg./rat on each succeeding day throughout the experiment. It has been established that the urinary radioactivity after the administration of labelled iodothyronine derivatives consists almost exclusively of iodide whilst that in the faeces is almost entirely due to thyronines (Gross & Leblond,

1951a, b; Roche, Michel & Tata, 1952; Wilkinson & MacLagan, 1953a). Consequently any differences between the urinary radioactivities of different groups must be a measure of variations in iodide output. It is clear from Fig. 1 that there is a marked reduction in the urinary iodide when BHDB is given along with triiodothyronine. Similar results

Fig. 1. The effect of BHDB upon the urinary radioactivity of rats treated with a single subcutaneous injection of ^{131}I -labelled triiodothyronine. $\odot$, the combined output of a group of three rats treated with triiodothyronine ($6\mu\text{g./rat}$); $\bullet$, the combined output of a group of three similarly treated rats given in addition daily oral doses of BHDB (25 mg./rat).

Table 1. The effect of BHDB on the urinary radioactivity of rats treated with ^{131}I -labelled triiodothyronine

Each row of figures refers to the combined output of a group of three rats. See text for dosage of BHDB.

Dose of triiodothyronine ($\mu\text{g./rat}$)	BHDB	Percentage of dose excreted after		
		24 hr.	48 hr.	120 hr.
30	Absent	11.0	18.4	20.6
		23.2	31.6	34.0
30	Present	2.8	6.3	9.0
		3.5	8.0	10.5
50	Absent	14.2	18.3	20.2
		20.3	25.8	28.2
		20.6	25.2	27.8
		19.2	23.7	26.8
50	Present	5.5	10.1	14.6
		3.3	5.9	8.3
		2.5	6.6	9.3
		5.4	*	*

* The metabolism cage containing this group of rats was damaged during the second day of the experiment and the urine became contaminated with faeces.

were obtained when doses of $30\mu\text{g.}$ triiodothyronine/rat were given (Table 1).

In order to exclude the possibility that the results were due to chance individual variations in the animals used in the experiment, the rats were kept for 5 weeks after which no radioactivity could be detected in the urine. Another experiment using $50\mu\text{g.}$ doses of triiodothyronine was then performed. In this case rats previously given BHDB + triiodothyronine were given triiodothyronine alone, and those which originally had the hormone alone now received both drugs. The results which appear in Table 1 show a depression of urinary iodide similar to those obtained in the first experiments.

In all three experiments the diminished urinary iodide loss in the group treated with BHDB was balanced by an increased faecal radioactivity due to thyronine derivatives (MacLagan & Wilkinson, 1954). There was thus no significant difference between the total excretion of labelled compounds in the two groups.

DISCUSSION

The suggestion that BHDB might owe its metabolic activity to interference with processes of deiodination was made by MacLagan *et al.* (1952) to explain the difference in its effects on mice treated with triiodothyronine and thyroxine. That it reduces the output of urinary iodide of rats injected with thyroxine was shown by Wilkinson & MacLagan (1953a) and Wilkinson *et al.* (1954). The present work completes this aspect of the study by showing that the output of urinary iodide is also reduced in similar experiments with triiodothyronine. Since BHDB has no effect upon the urinary excretion of injected iodide (Wilkinson *et al.* 1954), these reductions in iodide output must be a measure of the change in the degree of deiodination. The present observations, therefore, are in accord with our hypothesis, at least in intact rats. In thyroidectomized rats, however, Roche, Deltour & Michel (1953) have reported that BHDB increased the rate of deiodination of thyroxine labelled in the 3':5' positions with ^{131}I . We are not in a position to offer any explanation of this apparent contradiction, but it is conceivable that the metabolism of BHDB may differ in intact and thyroidectomized animals.

The possibility that the metabolic effects of BHDB might be due to alterations in the total rate of excretion of thyroxine or triiodothyronine has been considered, but is unlikely in view of our results. Thus in the experiments reported it was found that the lowered urinary excretion of iodide in the groups treated with BHDB was balanced by a higher faecal excretion of thyronine derivatives, so that the overall rate was similar in both groups. It

follows that the action of BHDB in inhibiting the effect of thyroxine on the oxygen consumption of mice and its opposite action in the case of triiodothyronine (MacLagan *et al.* 1952) cannot have been due to changes in the total excretion rate. They must therefore reflect alterations in the activity of the circulating thyroid hormone, probably consequent upon inhibition of deiodination processes.

SUMMARY

1. Details are given for a method of preparation from 3:5-diiodo-L-thyronine of triiodothyronine labelled in the 3' position with ^{131}I . Radioactive triiodothyronine appears to be much more stable in solution at pH 8–9 than labelled thyroxine.

2. The labelled compound was injected into rats, with or without simultaneous administration of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB), and the radioactivity of the excreta was measured.

3. Administration of BHDB caused a marked reduction in the output of urinary iodide in intact rats after a single injection of labelled triiodothyronine at a number of different dose levels. This is interpreted as being due to interference with the deiodination of this compound.

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Studies in Carotenogenesis

13. THE CAROTENOIDS OF THE FLOWER PETALS OF *CALENDULA OFFICINALIS*

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The presence of β - and γ -carotenes, lycopene, violaxanthin and rubixanthin in *Calendula officinalis* (pot marigold) has already been reported in the early investigations of Zechmeister & Chohnoky (1932) and Kuhn & Grundmann (1934); lycopene was absent from the very yellow varieties (Zechmeister & Chohnoky, 1932). Preliminary tests on the petals of a variety of *C. officinalis* producing very rich dark orange flowers showed that they contained a complex mixture of carotenoids including some which had not previously been reported. The investigation was therefore continued in greater detail.

EXPERIMENTAL

Materials. Fully opened flowers were gathered from a garden in north-west Cheshire. The seeds were obtained from Messrs Elsoms, Ltd., Spalding.

Extraction of pigments. The flower petals were removed from the calyx, placed in a Waring Blendor, covered with acetone and blended for 2 min. Most of the acetone extract was decanted, and the remainder removed from the residue by filtration through a sintered glass funnel. The residue was then returned to the Blendor and extracted again with acetone. This procedure was repeated until all the pigments had been extracted. The acetone extracts were combined and an equal volume of ether was added, followed by water