CLXXII. THE IDENTITY OF THE INDOPHENOL-REDUCING SUBSTANCE IN THE JENSEN RAT SARCOMA.

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A number of recent investigations carried out in this laboratory offered the opportunity of adapting the biological and spectrographic procedures to the co-ordination of the *l*-ascorbic acid content with the indophenol-reducing capacity of certain animal tissues. As conflicting views exist concerning the identity of the reducing substance or substances present in tumours (for historical details see following paper [Boyland, 1936]), it was considered by us desirable to utilize this technique in attempting to obtain more precise information. This was made possible by the kindness of Dr E. Boyland of the Research Institute of the Royal Cancer Hospital who generously consented to supply us with the great quantity of material necessary for the investigation. Our results seem to suggest that almost the entire if not all the indophenol-reducing capacity of the Jensen rat sarcoma is due to *l*-ascorbic acid.

Technique.

The experimental material. We are indebted to Dr Boyland for the following details. Small fragments of healthy Jensen rat sarcoma tissue were implanted with a small trochar needle under the skin in the flank of young rats under 120 g. in weight. The rats employed were either of the Wistar strain or of the "Middlesex Hospital" strain of tumour-susceptible rats. When the tumours had grown to a suitable size (4–8 g.) the tumour-bearing animals were killed by a blow on the head. The abdominal skin was cut and the tumour removed with forceps without opening the peritoneum. The tumours were removed daily for the biological test and were dispatched immediately to the Lister Institute. Forty-eight tumours were prepared for this purpose.

The biological tests. Immediately on arrival at the Lister Institute all the necrotic tissue was completely removed from the tumours. The remaining material was first ground with half its weight of powdered glass, and 60–100% of its weight of freshly glass-distilled water was then added to the mixture which was thoroughly ground again. The somewhat coloured and turbid aqueous extract was next separated from the solid tissue by centrifuging. In order to determine the indophenol-reducing capacity of the preparation with the greatest possible accuracy a part of the extract was precipitated with 3 volumes of 5% trichloroacetic acid, centrifuged and the clear colourless supernatant fluid titrated with the indicator at *p*H 2·5. The figures thus obtained were in fair agreement with those recorded by us in the original extract before treatment with trichloro-acetic acid. The aqueous preparation was then administered to the experimental guinea-pigs in doses, the indophenol-reducing capacity of which corresponded to

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0·25 and 0·5 mg. of *l*-ascorbic acid respectively. The administration of the freshly prepared doses, which was carried out daily, began after the animals had subsisted for ten days on a scorbutic diet. The time which elapsed between the removal of the tumour and the dosing of the extract was about 2 hours. A trichloroacetic acid extract of the aqueous preparation was made and titrated in most cases also at the end of the dosing, but no significant loss in the indophenol-reducing capacity of the extract was noted. The animals were killed by chloroform 20 days after the commencement of the dosing and the degree of scurvy if any was assessed at the post-mortem examination. Daily doses of 0·25 and 0·5 mg. of *l*-ascorbic acid were administered to control groups of guinea-pigs.

The spectrographic examination. An aqueous extract of the tissue was prepared as above except that when the indophenol-reducing capacity was not high a suitably smaller quantity of freshly glass-distilled water was used. This extract was then treated with 8·6 parts by volume of absolute alcohol and 0·4 part by volume of 2·5% $CdCl_2$ in 90% alcohol. After removing the precipitate by centrifuging, the centrifugate was further diluted with an equal volume of

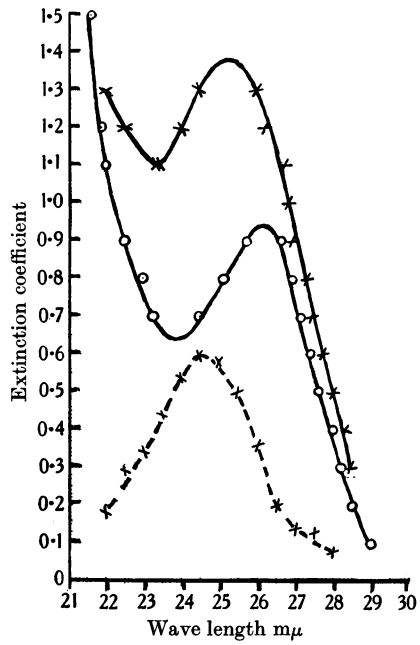

Fig. 1. Spectrographic results. The *l*-ascorbic acid content of this extract as calculated from the indophenol titration should have been 21·6 mg./100 ml. of extract. The above extinction coefficient corresponds to 21·5 mg./100 ml. of extract.

× — × Tumour extract. o—o Tumour extract after treatment with Cu.
× - - - × Ascorbic acid by difference.

absolute alcohol. One portion of this mixture (10 ml.) was then acidified with hydrochloric acid so as to give a final concentration of 0·02 *N* HCl and immediately compared in the spectrograph against a blank solution. To another 10 ml. of the above mixture 1 drop of 1% $CuSO_4$, $5H_2O$ solution was added and the mixture allowed to remain for 2 hours. It was then acidified as before and examined against a control (see Fig. 1). This latter treatment destroys *l*-ascorbic acid entirely

when present in concentrations such as were encountered in these experiments, and consequently it could act as an index for the probable identity of the vitamin with the substance showing a point of maximum absorption at 245 $m\mu$. Details of the spectrograph have already been given by us [Kellie & Zilva, 1936]. A 1 cm. absorption tube was used. The content of ascorbic acid was calculated from the extinction coefficient, since the concentrations were adjusted so as to fall within the limits in which Beer's law holds true [cf. Herbert *et al.* 1933]. The molecular extinction coefficient as determined by us for pure *l*-ascorbic acid in acid alcohol solution was found to be 10,000, and this value was therefore used in the calculation of the results.

Results.

Fig. 2, which gives the graphic representation of the biological tests, shows that the responses of the animals on the *l*-ascorbic acid doses and of those which received the equivalent doses of tumour extract as assessed by indophenol titration are the same. From Table I we see further that the average of 15 spectrographic determinations accounts for 78·8 % of the *l*-ascorbic acid as measured

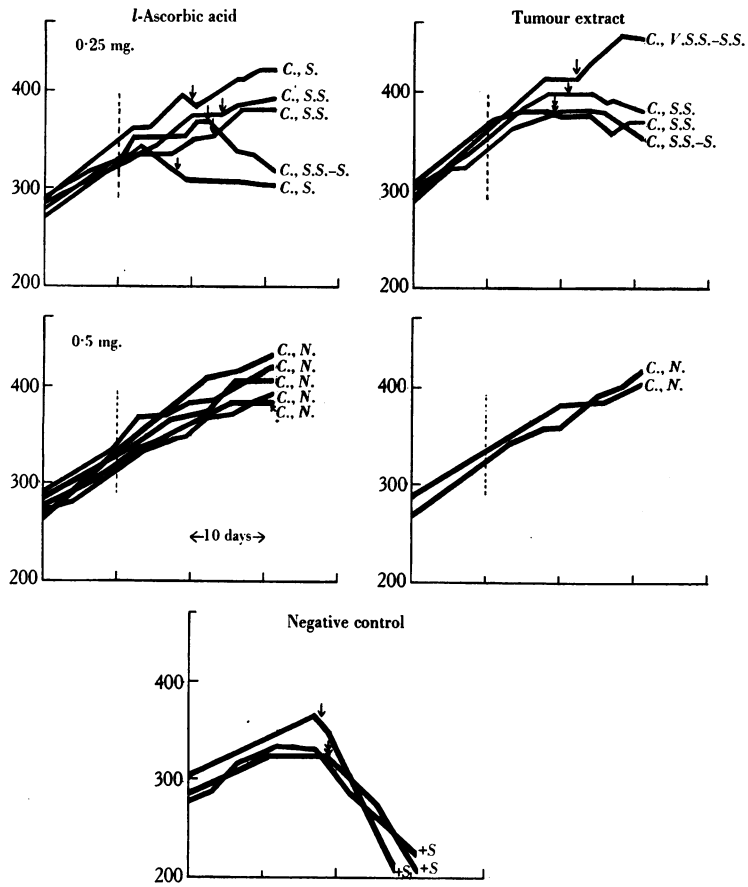

Fig. 2. Biological tests.

⁞ = beginning of dosing; ↓ = onset of clinical symptoms of scurvy; + = died; *C.* = chloroformed; *N.* = normal; *V.S.S.* = very slight scurvy; *S.S.* = slight scurvy; *S.* = scurvy.

Table I.

Ascorbic acid calculated from indophenol titration mg./100 ml. extract	Ascorbic acid found spectrographically mg./100 ml. extract	%
21·6	21·5	100
18·1	12·9	71
16·7	10·8	65
17·1	15·2	71
16·7	15·6	93
23·4	21·8	93
15·3	11·5	75
24·0	17·6	73
16·1 }	12·2 }	76 }
16·1 }	12·6 }	78 }
18·1	15·2	84
10·0	6·7	67
22·0	17·8	81
28·6 }	21·6 }	75 }
28·0 }	22·7 }	81 }
Average —	—	78·8

The bracketed figures refer to determinations carried out on the same extract within an hour of one another.

titrimetrically. That the characteristic absorption is due to *l*-ascorbic acid and not to chemically related compounds in this case is evident from the biological and indophenol tests. The average figures obtained by spectrographic measurements are, however, undoubtedly low. A difference of 21 % or even less would have been reflected in the condition of the animals, especially of those on the higher doses in the biological determination. It is also necessary to consider the fact in this connexion that figures tend to be lower than their real value in spectrophotometric assessments when concentrations of ascorbic acid in physiological fluids as low as those above are examined. This is illustrated by the experiment (Table II) in which plasma was submitted to the same precipitation treatment

Table II.

Ascorbic acid added mg./100 ml. plasma	Ascorbic acid found spectrographically mg./100 ml. plasma	%
24·9	22·4	90
29·4	24·6	84
30·3	29·0	96
24·9	22·8	92
Average —	—	90·5

with alcohol and $CdCl_2$ as the tumour extracts, and in which a known quantity of *l*-ascorbic acid of a similar order to the above was added to the final solution immediately before the spectrographic examination. An average of only 90·5 % of the added ascorbic acid is accounted for here. This error would be expected to be greater with tumour extracts in which the ascorbic acid is present before precipitation. It is of importance to note that in spite of the average of 78·8 % recorded by the spectrograph for these extracts, as high a figure as 100 % has been obtained (cf. Fig. 2), and that the average of the determinations in the case of tumours with a high content above 20 mg./100 ml. of extract, when the in-

herent error of the method would be expected to be less, was as high as 83·8%. These facts suggest that almost the entire indophenol-reducing capacity of aqueous extracts of Jensen rat sarcoma is due to *l*-ascorbic acid.

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