MICROBIOLOGICAL ASSAY OF THE OVERALL POTENCY OF GROWTH-FACTORS OF SHARK LIVER EXTRACTS

BY

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LIVER is reputed to constitute one of the richest sources of the B-complex, the anti-ænemic principles and other unidentified growth-factors. Williams *et al.*,[1] in their study of pantothenic acid, used liver as the starting material. Kuhn and Wieland[2] have shown that tunny fish liver contains the same active principles as those which characterise mammalian livers.

With the development of the shark liver oil industry in India, appreciable quantities of liver residues are, at the moment, available at various centres of the industry. It was, therefore, of interest to make a systematic study of this potentially useful bye-product, with special reference to its content of the B-complex. The present communication deals with a microbiological assay of the overall potency of the growth-factors of the liver extracts as revealed by the response potentiated by varying doses of the extract towards a strain of *Lactobacillus* (Culture No. 3, N.C.T.C.: 2078).

Research Material

The three commercially known varieties of sharks generally worked up for their oil were selected for the investigation. Table I gives data regarding the contents of their oil and its vitamin A value as determined by one of us (I. M. G.).

Table I

Zoological Name	Local Name	Oil content per cent.	Vit. A in I. U./gm.
1. *Carcharinus melanopterus*	Khada (K)	50·7	55600
2. *Galeocerdo tigrinus*	Waghbeer (W)	70·0	4700
3. *Carcharinus limbatus*	Pisori (P)	40·9	9700

Preparation of Extracts

The liver residue (100 gms.) in each case was treated with 100 ml. of water and the mash digested at pH 5·5 with activated papain (5 gms.) at 40° C. for 48 hours. The digest was filtered, pH of the filtrate adjusted to 4·5 with acetic acid, steamed for an hour and the precipitate filtered off. The pH of the filtrate was carefully readjusted to 7·0 with 10 per cent. sodium hydroxide. The extract was afterwards distributed into 10 ml. ampoules which after sealing were sterilised at 15 lbs. pressure for 20 minutes. In the case of the extract from sheep's liver, digestion with papain was preceded by autolysis at 30° C. for 48 hours. For comparison, Lily liver extract (I.U.S.P. unit/ml.) was employed as the standard.

Table II

Liver extract	Milligrams per milli litre of extract			Complexity
	Total solids	Total N	Amino N	
Lily (L)	151·0	15·6	3·24	4·81
Sheep (S)	330·0	28·6	9·12	3·13
Khada (K).	115·2	16·0	4·51	3·55
Waghbeer (W)	111·0	12·9	3·98	3·24
Pisori (P)	119·5	16·8	3·48	4·82

Selection of the Organism and the Preparation of the Inoculum

The organism (*Lactobacillus*, strain No. 3, N.C.T.C.: 2078) employed for the microbiological assay, was obtained from the National Collection of Type Culture, India, Indian Institute of Science, Bangalore. Consequent to a comprehensive study of the nutritional requirements of the lactic group of organisms in the National Collection, this strain was found to give the maximum response to most of the vitamins of the B-complex so far examined.

Stab cultures of the organisms were carried in liver extract.—Pentone, glucose, agar (3 ml. 100 ml., 0·5 per cent., 1 per cent., 1·5 per cent.). The method of preparing and carrying cultures were those described by Snell and Strong.[3]

Inoculum for assay tubes was prepared by transfer from the stock culture to a sterile tube of basal medium to which sheep liver extract was added (3 ml./100 c.c. of B.M.). The inoculum was incubated at 37° C. for 24-36 hours before use.

Basal Medium

A simple synthetic basal medium (B.M.) was selected and is a modification of that used by Snell and Strong,[3] for the determination of riboflavin, and by Pennington, Snell and Williams[4] for the determination of pantothenic acid. It contains acid-hydrolysed casein (vitamin and fat-free) 0·5 per cent., tryptophane 0·01 per cent., *l*-cystine 0·01 per cent., glucose 4·0 per cent., sodium acetate 2·4 per cent., and inorganic salts. The constituents are prepared and preserved as follows:—

Acid-Hydrolysed Casein.—50 Gms. of vitamin and fat-free casein (B.D.H.) were hydrolysed with 260 ml. of 25 per cent. H_2SO_4. The mixture was autoclaved for 10 hours at 15 lbs. SO_4-ions were removed with $Ba(OH)_2$ and any excess Ba-ion was carefully removed with the minimum amount of H_2SO_4. The solution was adjusted to contain 100 mg. of dry matter per ml. It was preserved under toluene. Traces of vitamins were effectively removed from the casein hydrolysate with 10 mg./ml. of 'Norit' at pH 3·0.

Tryptophane and Cystine.—Stock solutions of tryptophane and cystine hydrochloride containing 1 mg./ml. were prepared and kept under toluene.

Inorganic Salts.—Solution A contains 25 gms. of KH_2PO_4 and 25 gms. of K_2HPO_4 dissolved in 250 ml. of water.

Solution B contains 10 gms. of $MgSO_4.7H_2O$, 0·5 gm. of NaCl, 0·5 gm. of $FeSO_4$. $7H_2O$ dissolved in 250 ml. of water. Five drops of concentrated HCl were added to stabilise the solution. 0·5 Ml. of soltion A and 0·5 ml. of solution B contain the requisite inorganic salts for 100 c.c. of basal medium.

Assay Procedure

A medium having 2·5 times the concentration of the basal medium was prepared, pH adjusted to 6·8 and 2 ml. of this medium were transferred into each assay tube. Graded doses of liver extracts corresponding to 0·021, 0·42, 0·083, 0·208, 0·417, 0·625, 1·25, 1·875, 2·5, 3·0, 3·75 and 4·25 mg. of nitrogen were added and in each case, sufficient distilled water added to bring the final volume in each tube to 5 ml. Duplicates and a blank were run for each concentration. The tubes were sterilised at 10 lbs. for 30 minutes, twice at an interval of 24 hours. Three loops of the inoculum were inoculated into each assay tube and incubated at 37° C. for 72 hours. The acidity produced during this period was directly titrated against 0·1 N NaOH to pH 6·8-7·0 using bromthymol-blue as indicator. Results are given in Table III and are also graphically represented (Fig. 1).

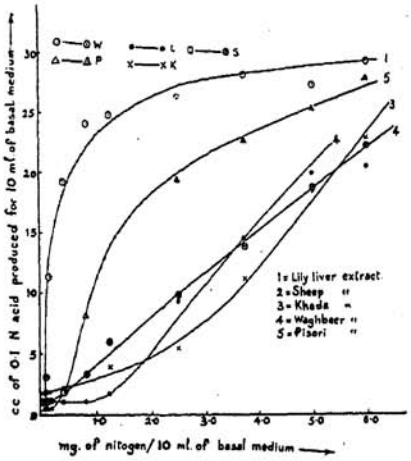

FIG. 1. Responses of N.C.T.C. 2078 to Liver Extracts

A close study of Table III and Figure 1 will reveal that, the Lily liver extract, per milligram of nitrogen potentiates the maximum response. Next in order comes the liver extract of Pisori, which, from the nature of the curve may be suspected to have a vitaminic make-up closely approximating to that possessed by the Lily liver extract. In other words, the functional similarity of the two extracts as revealed by the curves (see Fig. 1) suggests that Pisori liver might constitute a rich source of the antianæmic principles. The extracts from the livers like Khada and Waghbeer exhibit comparatively poor potencies; sheep's liver extract, however, gives a steady and linear response for the entire range of concentrations studied.

The minimal concentration of the extract (in terms of total nitrogen) at which the potency of the extract tends to get abolished is different for each of the extracts. These critical

TABLE III

Results expressed as ml. of 0·1 N acid produced for 10 ml. of basal medium

Liver Extract	Concentration of extract in mg. of nitrogen in 10 ml. of basal medium.												
	0·042	0·083	0·125	0·167	0·417	0·834	1·250	2·500	3·750	5·00	6·00	7·50	8·50
Lily	1·1	1·1	3·1	11·3	19·3	24·1	24·7	26·3	28·0	27·2	29·0	32·1	34·3
Sheep	1·0	1·0	1·0	1·0	1·8	3·4	5·1	9·8	13·8	18·6	22·0	26·8	29·7
Khada	1·2	1·2	1·5	1·9	2·2	3·3	3·9	5·4	11·2	18·4	22·6	24·1	27·9
Waghbeer	1·1	1·1	1·1	1·1	1·1	1·1	1·8	9·4	14·5	19·9	20·3	22·5	29·7
Pisori	0·4	0·4	0·4	0·4	1·8	8·2	14·2	19·3	22·6	25·2	27·6	29·5	32·7

concentrations which are underlined in the table (Table III) represent the point below which the growth-factors, singly or severally, attain their respective limiting concentrations. It is interesting to observe that this limiting concentration is reached at the lowest nitrogen level (0·125 gms.) in the case of the Lily liver extract while in the case of Waghbeer liver extract the point is attained at a higher level of nitrogen (1,250 mgms.). The other extracts, including that of Pisori, exhibit loss of potency at a level of nitrogen corresponding to 0·417 milligrams.

It can also be noted that the response to the addition of the next higher concentration of the extract (higher than the critical concentration) is usually quick and substantial, particularly in the case of Lily and Pisori extracts.

The limiting concentrations of nitrogen may roughly be taken to be inversely proportional to the degree of purity of the extracts with respect to the growth-promoting factors. The potency and purity of the extracts may also be computed from the levels of nitrogen at which a given response is potentiated. For example, a response equivalent to about 10 ml. of decinormal alkali is given by 0·150 mgm. of Lily extract, 2·5 mgms. of sheep's, 3·7 mgms. of Khada's, 2·9 of Waghbeer's and 0·95 of Pisori's (computed from the curves, see Fig. 1). Taking Lily liver extract as containing 100 units of overall potency, the potencies for the extracts of sheep, Khada, Waghbeer and Pisori respectively work out as 6, 4, 5 and 16.

It has previously been suggested that the critical limiting value may have been reached with respect to a single or a multiple vitamin factor. With a view to elucidate this point, experiments were conducted, each of extracts being employed at the respective limiting level of nitrogen. Crystalline vitamins, thiamin, riboflavin, niacin, pyridoxine, calcium pantothenate and inositol were tried. Results are given in Table IV.

TABLE IV

Medium	Ml. of 0·1 N Acid produced for 10 ml. of B. M.		
	Khada	Waghbeer	Pisori
1. B. M. + L. E. + all vitamins	17·2	15·2	12·0
2. B. M. + L. E. + all vitamins except thiamin	15·7	14·1	11·0
3. B. M. + L. E. + all vitamins except riboflavin	8·4	14·0	10·6
4. B. M. + L. E. + all vitamins except Pyridoxin	17·4	15·5	10·6
5. B. M. + L. E. + all vitamins except Pantothenate	12·6	4·4	5·5
6. B. M. + L. E. + all vitamins except niacin	17·6	15·5	12·0
7. B. M. + L. E. + all vitamins except inositol	17·6	15·5	12·0

B.M. = Basal Medium; concentration of the vitamins used for each of the assay tubes was as follows:—Thiamin 0·5 γ; niacin 0·5 γ, calcium pantothenate 1 γ, riboflavin 1 γ, pyridoxine 0·5 mgm. Liver extract (L.E.) added = the limiting level of nitrogen as indicated in Table III.

Figures given in Table IV are highly significant; they suggest that the limiting concentration is reached mainly with respect to one of the vitamins. Khada lacks riboflavin mainly while pantothenic acid appears to be lacking in the liver extracts of Waghbeer and Pisori. The potencies of the extracts can, therefore, be effectively increased by the addition of the deficient vitamins.

SUMMARY

1. A comparative microbiological assay of the overall potency of the growth-factors of liver extracts, has been carried out using a strain of lactobacillus culture (N.C.T.C.: 2078), as the test organism. Three varieties of sharks, *Carcharinus melanopterus, Galeocerdo tigrinus, Carcharinus limbatus*, whose liver residues constitute a useful bye-product of the shark liver oil industry, have been investigated.

2. *Carcharinus limbatus* has been found to yield the most potent extract with a functional similarity approximating to the standard Lily liver extract.

3. Shark livers with high contents of the fat-soluble vitamins appear to constitute poor sources of the water-soluble B-complex. The two groups of vitamins appear to occur together in the inverse ratio (see Table I).

4. Recent clinical reports suggest that the antianæmic factors (extrinsic) are identifiable with some of the important members of the B-complex. Further, recent studies have shown that the B-vitamins are essential for the synthesis of hæmoglobin and the formation of corpuscles. These observations are significant in relation to the functional behaviour of the Pisori liver extract and suggest that it might prove a good source of the antianæmic factors.

5. The response of the various extracts at their limiting concentrations to the addition of specific vitamin shows that some of the vitamins are lacking in them; the addition should serve to render the extract physiologically more balanced and potent.

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1. William, R. J., *et al.*, *J. Amer. Chem. Soc.*, 1939, **61**, 454; *Ibid.*, 1939, **60**, 2719. 2. Kuhn, R., and Wieland, T., *Ber.*, 1940, **73B**, 962. 3. Snell, E. E., and Strong, F. M., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 346. 4. Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.* 1940, **135**, 213.

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