Biochemical Studies on the Liquefaction of Fish Body—III. On Effectiveness of Fish-Autolysate to the Culture of Bacteria

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It has been an urgent problem in Japan to prepare a substitute for LIEBIG meat extract by making use of fishery products, since we have a poor resource of live stocks. Many studies have been carried out with hopeful results.

As early as in 1900, it has been shown by TOZUKA¹⁾ that a number of bacteria were able to grow normally on a hot watery extract of either fresh fish or its dried product to a similar extent on the meat extract. Any difference in growth rate of bacteria has not been observed between squid extract and Liebig extract²⁾. An extract of oyster³⁾ or Laminaria⁴⁾ has also been found to be comparable to the meat extract. HIDAHA⁵⁾ and ARIZAWA⁶⁾ have reported that a good growth response of bacteria similar to that on Liebig extract was obtained on a hot watery extract of bonito which is a by-product in making the dried bonito. TAKEBAYASHI⁷⁾, however, has indicated that it is inadequate for the preservation of strain or isolation of new species without the addition of an alcoholic extract of the rice bran. IITOYO⁸⁾ has shown that an extract of fish, if used instead of peptone, was found to be an appropriate culture medium for *Vibrio cholerae*. KIMATA⁹⁾ has observed that a hot watery extract of fish meal could also be used without any inferior result than LIEBIG extract. ARIMA¹⁰⁾, and TOYAMA and YASUKAWA¹¹⁾ have carried out detailed experiments showing that a warm watery extract of sardine prepared according to OGURA's procedure gave a little better result than LIEBIG extract in the rate of growth and indol- and toxin-formation of several pathogenic bacteria. A similar result has been obtained by a hot watery extract of sardine¹²⁾. TSUKAHARA¹³⁾ has reported that a digest of whale meat by pepsin was more effective than LIEBIG extract, whereas a hydrolysate of the whale meat with 20 per cent hydrochloric acid was less effective.

It has intensively been studied in our laboratory during World war II to prepare an effective culture media by the use of fishes as its material. A success was finally made in getting a mixture of peptone and amino acids in an industrial scale by subjecting whole fish body to autolysis under a certain condition¹⁴⁾. It has been found that the preparation is very rich in ingredients which promote the growth of bacteria. It is quite a striking fact that just a simple solution of fish-autolysate without add-

ing anything else is very effective to the culture of *Corynebacterium diphtheriae* as well as *Streptococcus hemolyticus* which are not ordinarily able to grow on the usual culture media. The chemical nature of the growth factor of the preparation is under investigation.

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I. Method and Materials for the Experiment

1. The preparation of fish-autolysate and its composition

The fish-autolysate was obtained by subjecting whole fish body to the autolysis at about 50°C. and in slightly acidic reaction¹⁴⁾. The liquefied part after being neutralized was clarified and concentrated to a syrup and then dried in a desiccator under reduced pressure. The powdered preparation has the following composition on average : moisture, 5 per cent ; nitrogen, 13 per cent ; ash, 9 per cent. The fractionation of various forms of the nitrogen according to WASTENEYS and BORSOOK's procedure¹⁵⁾ gave, on average, values shown in Table 1.

Table 1. The analytical result of the fish-autolysate.

	Fish-autolysate		A commercial peptone	
	Per cent Total nitrogen	Per cent dry matter	Per cent total nitrogen	Per cent dry matter
Protein- and metaprotein-N	1.91		1.21	
Proteose-N	11.80		31.38	
Peptene-N	20.39		6.18	
Subpeptone-N	26.00		} 61.23 ⁺	
Amino acid-N	39.90			
Total nitrogen		12.98		12.74*
Ammonia		0.365		
Creatinine		2.12		
Ash		13.82		
Calcium		2.88		
Magnesium		0.089		
Chloride		7.75		

* The nitrogen contents of polypeptone (Takeda Co.), Teruuchi-peptone and a peptone supplied by Katayama Co. were 12.7 %, 12.0 % and 12.1 %, respectively.

+ Sum of subpeptone-N and amino acid-N.

2. The experimental method

Several species of bacteria mainly pathogenic were used for the experiment. A simple two per cent solution of fish-autolysate was used for the preparation of the liquid media and the agar plate media. As control media two kinds of the usual media were used, namely, one containing one gram of the extractive of bonito and one gram of the Teruuchi-peptone per 100 cc., and the other containing one gram

of the LIEBIG extract and one gram of the Teruuchi-peptone per 100 cc. It has been noticed that, while egg white is ordinarily needed to clarify the agar media, the present preparation can be used without any process of clarification.

The comparison between the present preparation and the usual media was made, on one hand, by the turbidity as well as the intensity of the indol reaction in case of culture in liquid media, and, on the other, by an outer appearance of colonies developed on the agar plate media.

II. Experimental Results

1. The growth in liquid media and on plate media

The results are shown in Tables 2 and 3. From these findings it will be clear that the present preparation is superior to the control media. It has also been noticed that *B. pyocyaneus* cultured by the fish-autolysate forms pyocyanin in a remarkable quantity not comparable to the control media. Furthermore, the color reaction due to indol was observed to be much more distinguishable than that of the control media,

Table 2. The growth of several bacteria in liquid media observed after a 24-hour incubation at 37°.

Species of bacteria	Kinds of culture media*	Turbidity	Indol reaction	
			KITASATO-SALKOWSKI	PRINGSHEIM
<i>E. coli</i>	(a)	++	±	++
	(b)	++	—	+
	(c)	++	—	+
<i>Staphyl. aureus</i>	(a)	++	—	+
	(b)	++	—	+
	(c)	++	—	+
<i>Bac. proteus</i>	(a)	++	—	+
	(b)	+	—	+
	(c)	+	—	+
<i>Bac. subtilis</i>	(a)	++	—	—
	(b)	+	—	—
	(c)	++	—	—
<i>Bac. pyocyaneus</i>	(a)	++		
	(b)	++		
	(c)	++		
<i>Corynebacterium diphtheriae</i>	(a)	++		
	(b)	—		
	(c)	—		
	(d)	—		
	(e)	—		
	(f)	—		
	(g)	—		
	(h)	—		

* Per 100 cc. solution, media (a) contains 1 g. of the fish-autolysate and 1 g. of the Teruuchi peptone ; media (b) contains 1 g. of a bonito-extract and 1 g. of the peptone ; media (c) contains 1 g. of the LIEBIG extract and 1 g. of the peptone ; media (d) contains 2 g. of the LIEBIG extract ; media (e) contains 1 g. of a fish-extract prepared according to OGURA's procedure and 1 g. of the peptone ; media (f) contains 2 g. of the fish-extract ; media (g) contains 2 g. of the peptone ; media (h) contains 2 g. of a peptone supplied by Katayama Co. The concentrations of these ingredients are all based on dry basis. All these media contain sodium chloride at a 0.3 per cent level.

Table 3. The growth of several bacteria on plate media observed after a 24-hour incubation at 37°

Species of bacteria	Colonies				Order of superiority	Species of bacteria	Colonies				Order of superiority
	Kinds of culture media*	Abundance	Largeness	Thickness			Kinds of culture media*	Abundance	Largeness	Thickness	
<i>E. coli</i>	(a)	##		++	1	<i>Vibrio cholerae</i>	(a)	++	##		1
	(b)	++		+	2		(b)	+	+		3
	(c)	+		+	3		(c)	+	++		2
	(d)	+	no difference				(d)	+		no difference	
	(e)	±					(e)	++			
	(f)	±					(f)	+			
	(g)	±					(g)	+			
	(h)	+					(h)	++			
<i>Staphyl. aureus</i>	(a)	++	##	##	1	<i>S. paratyphi</i> A	(a)	##	##	no difference	1
	(b)	++	++	##	2		(b)	+	+		3
	(c)	++	++	++	3		(c)	++	++		2
	(d)	++				<i>S. paratyphi</i> B	(a)	##	++	##	1
	(e)	+					(b)	+	+	+	3
	(f)	++					(c)	++	++	++	2
	(g)	+					(a)	##	++	##	1
	(h)	++					(b)	+	+	++	3
<i>Bac. proteus</i>	(a)	++	no difference	##	1	<i>Bac. typhi</i>	(c)	++	++	+	2
	(b)	+		++	2		(d)	+			
	(c)	+		+	3		(e)	++			
<i>Bac. subtilis</i>	(a)	##	no difference	no difference			(f)	+			
	(b)	##					(g)	+			
	(c)	##					(h)	++			
<i>Bac. pyocyaneus</i>	(a)	++	##	+	1	<i>Bac. dysenteriae</i>	(a)	++	##	no difference	1
	(b)	+	++	++	3		(b)	++	+		3
	(c)	+	++	##	2		(c)	++	++		2
<i>Strept. haemolyticus</i>	(a)	##			1		(d)	±			
	(b)				2		(e)	+			
	(c)	—					(f)	±			
							(g)	±			
							(h)	±			

* Notations of media are the same as those given in Table 2.

All these media contain agar—agar at a 3 per cent level.

probably being due to its high tryptophan content (1.0–1.3 per cent) and also to a pale coloration of liquid media.

2. some observations in culturing certain pathogenic bacteria

a) The rate of growth of *Vibrio cholerae*.

When 5 cc. of two per cent fish-autolysate were inoculated with *Vibrio cholerae* in an amount as minute as 1/100,000 a platinum-loopful quantity of the bacteria, a distinct turbidity and the cholera rose reaction appeared after a 5- to 6-hour incubation at 37°C. In the control experiment using LIEBIG extract, however, the reaction was positive only after a 24-hour incubation. This result indicates that an early diagnosis of cholera disease will be realized without any difficulty by use of the present preparation.

b) The culture of *Corynebacterium diphtheriae*.

The rate of growth of *C. diphtheriae* which was observed in the liquid media of the present preparation was two to three times greater than that in LOFFLER's or MARTIN's media ; a 24-hour incubation after innoculating to two per cent solution of the present preparation was enough to obtain a fairly good formation of the membrane on the surface of the liquid media.

The toxin which was obtained after being cultured through two successive generations by two per cent solution of the present preparation showed M. R. D. of 0.000001 cc. on being tested by RÖMER's method.

The volutin of the bacteria grown on the plate media can clearly be observed without fail after staining. Therefore, by the use of the preparation, it will become to be quite feasible to identify *C. diphtheriae* and to efficiently manufacture the anatoxin.

3. The change of growth-promoting activity during autolysis

From the point of view to prepare an efficient culture medium and further to elucidate the chemical nature of the growth-promoting ingredient, it will be of interest to see the change in the growth rate in relation to the autolytic degradation of fish body. The agar plate media which contained at a two per cent level the autoly-

Table 4. The change in growth-promoting activity with progress of autolysis.

[illegible]

Fig. 1. Change of molecular size of nitrogenous compounds with the progress of autolysis.

sates of sardine having different degrees of cleavage were inoculated with several kinds of bacteria. The qualitative differences in growth of the bacteria among these media are shown in Table 4. On the other hand, the changes of molecular size of nitrogenous compounds with the progress of the autolysis are shown in Fig. 1. It will clearly be seen that the growth-promoting activity shown by the autolysates varies in general with the progress of the autolysis. Such a difference in the activity, however, was hardly observed for *Staphylococcus aureus* and *Vibrio cholerae*.

The quantitative determination has been made of the growth rates of *Bac. typhi* and *E. coli* in the liquid media by comparing the turbidity due to the bacteria against an arbitrary standard made of a suspension of diatomaceous earth. Figs. 2 and 3 show that the autlysate obtained after 5~13-hour autolysis gave the maximum rate of growth for these two kinds of bacteria. This maximum activity appears to occur after the marked decrease in the proteose fraction. It needs a further study, however, to determine whether the peak in growth rate is due to that the growth factor might be available in maximum quantity or due to that the forms of nitrogen be suitable to the bacterial growth.

III. Summary

1) A preparation which is effective in culturing bacteria instead of LIEBIG extract and peptone has been obtained by autolyzing whole fish body.

Fig. 2. Rate of growth of *E. coli* in the autolysates having varying degree of autolysis.

Fig. 3. Rate of growth of *Bac. typhi* in the autolysates having varying degree of autolysis.

2) Experiments have shown that it is superior to the usual media when judged from the magnitude of colonies on the plate media and the turbidity of the liquid media

3) The liquid media gave a distinct indol reaction on culturing either *E. coli*, *Staphyl. aureus* or *Bac. proteus*. The liquid media gave the easily perceptible cholera rose reaction after a 6-hour incubation at 37°C. after inoculating *Vibrio cholerae* in a quantity as minute as 1/100,000 a loopful bacteria.

4) *Corynebacterium diphtheriae* was able to grow on a simple two per cent solution of the present preparation with a growth rate two to three times greater than on MARTIN or LOFFLER's media. The volutin of the bacteria grown on the preparation could be identified without fail on staining. The minimum skin reaction dosis of the toxin formed was 0.000001 cc.

5) It has been shown that the activity of promoting the growth of bacteria which was displayed by the preparation was in variance with the progress of the autolysis.

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