CCXLVI. EXPERIMENTS ON METABOLISM WITH *C. DIPHTHERIAE*. I.

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THE preparation of diphtheria toxin on a large scale is, in spite of the numerous investigations which have been carried out on this subject, still a very uncertain operation. Without doubt the cause of this must be sought to a considerable extent in the fact that the usual culture media are of a very complicated nature and always contain besides the simple compounds such as sugars, inorganic salts etc., meat extract and one or other of the various kinds of peptone.

Most of the experiments on the dissimilative metabolism of *C. diphtheriae* mentioned in the literature have been carried out on media of much simpler composition.

It is a striking fact, however, that in all these experiments little or no attention is paid to the formation of toxin as such. Only a few investigators make any mention of this, yet as a rule the quantity of toxin formed is so small as to be of no practical value.

In both the older and the more recent literature there is a very large number of communications in which different "routine" culture media are described and attention is drawn to some of the important factors affecting toxin formation. The older literature is conveniently summarized in the monograph "Diphtheria" published by the Medical Research Council [1923]. After an extensive discussion of all the data then available the authors come to the conclusion: "At the moment it is only by adhering rigidly to certain formulae of technique that one may hope to arrive at a satisfactory product and, even with the most careful work and attention to detail, some batches of toxin do not come up to the standard of potency demanded by the maker of antitoxin. Certain factors are still beyond control, and until these are elucidated, the problem of making toxin on an entirely satisfactory basis will not be solved."

In spite of the very great amount of work which has been carried out on this subject since 1923, the verdict pronounced above still holds in its entirety.

A very extensive study on the formation of toxin was carried out by Pope [1932]. He started from a so-called "semi-synthetic" medium, in which Difco-proteose-peptone was employed as the source of nitrogen in conjunction with a few inorganic salts. Various sources of carbon such as sugars, organic acids etc., were added to a relatively simple "standard solution" and the effects of these additions on the formation of toxin investigated. Further attention must be drawn to a publication by Ström [1935]. This investigator examined the influence of various substances, when added to a simple stock solution, on the course of pH and the formation of toxin after inoculation with a diphtheria culture. The problem of the biochemistry of toxin production will be elucidated only when the following conditions are fulfilled.

1. The "stock solution" used must be as simple as possible; for this purpose the "semi-synthetic" medium described by Pope is eminently suitable.

2. An attempt must be made not only to obtain a qualitative, but as far as is possible, a quantitative idea of the processes involved.

3. The experiments must be carried out in direct connexion with toxin formation. Thus a medium of simple composition may be chosen, but a definite amount of toxin must be formed in this medium during incubation after inoculation with a diphtheria culture. Experiments in which no toxin is formed may of course be of value as supplementary evidence or as controls, but they are not essential.

In this communication we shall describe our experiments on the questions how rapidly sugars (glucose and maltose) are decomposed by diphtheria bacilli when added to a simple culture medium, and how this decomposition affects pH and the formation of toxin.

Method of experiment.

As culture medium use was made of the "semi-synthetic" medium of Pope, having the following composition:

Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.2 g.
Calcium chloride ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$)	0.1 g.
Sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)	1.0 g.
Potassium phosphate (K_2HPO_4)	1.0 g.
Glacial acetic acid	3.0 ml.
Difco proteose-peptone	20.0 g.
Distilled water	1000 ml.

All the constituents were dissolved without heating, pH was adjusted to 8 with 25 % NaOH solution and the sugar (glucose or maltose) added. The resulting solution was then filtered through paper and sterilized by filtration through a Seitz Entkeimungs-filter.

Culturing was carried out in large Fernbach flasks, having a diameter of 40 cm. and a height of 7 cm. Two litres of liquid culture medium were used in each flask. The liquid layer had a thickness of 2 cm. so that there was a very favourable ratio between surface and the thickness of the layer. The very wide necks of the flasks were closed by means of cotton-wool plugs carrying a tube about 12 mm. in diameter. After the desired quantity of sterile liquid had been introduced into the flask, this tube was closed by means of a rubber stopper through which a glass siphon tube passed. By means of this tube, fitted with a rubber tube, pinchcock, and glass protector, a sample could be taken at any desired time under sterile conditions. The sterility of the solution was checked by incubating the medium for two or three days before inoculating.

The strain used was the well-known Park-Williams 8, which is regularly used in this laboratory and which we obtained initially from Tomcsik. A day before the experiment, the strain was subcultured into fresh Löffler serum tubes and the 24-hour old culture suspended in salt solution and inoculated into the various flasks. Incubation took place at 35–36°.

Sampling was carried out as follows. First of all about 8 ml. of liquid were collected in a sterile measuring cylinder (10 ml. capacity) and, this being the volume of the siphon, it was thrown away. The necessary amount of liquid was then siphoned into a larger sterile measuring cylinder.

The following points may be mentioned as regards the methods of analysis. The glucose and maltose determinations were carried out by the method of Luff described in detail by Schoorl [1929; 1930], after the peptone had been precipitated with phosphotungstic acid [see Seibert, 1926; Tasman & Pot, 1934]. The 2 % peptone solution used showed a very small amount of reduction with the

sugar test solution, which was included in each determination as a blank correction.

The pH was determined with a hydrogen electrode against a saturated calomel electrode, in an apparatus as used by Smit [1928].

The formation of toxin was examined by means of Ramon's [1923] flocculation test carried out at 50° and expressed as $\frac{1}{Lf}$, determined against a serum dilution containing 100 antitoxin units per ml. By making use of 5 ml. quantities of toxin in each flocculation tube, a difference of 0.2 in the $\frac{1}{Lf}$ value can be determined with certainty for values of $\frac{1}{Lf}$ below 3.

All the experiments described in this paper were carried out at least in duplicate. Taken on the whole, the agreement between parallel experiments was very good. Only one experiment of each type is quoted.

In the first place, the above-mentioned values were determined in the Pope bouillon containing sodium acetate formed during the neutralization of the acetic acid. The results are given in Fig. 1.

Fig. 1 (Exp. 1). Pope medium with acetate and 0.2% glucose. A, Glucose; B, pH ; C, $1/Lf$.

From these results it appears that the glucose disappears from the liquid in about 34 hours, whilst the formation of toxin begins before this time. The formation of toxin takes place at a practically constant rate until the maximum is reached, and after this time no further increase in the $\frac{1}{Lf}$ value occurs. In all our experiments we have noted the same behaviour. In the early stages, the hydrogen ion concentration follows the decomposition of the sugar. As soon as the latter has disappeared, the well-known reversal occurs, the pH rises, rapidly at first and then more slowly. A possible explanation of this behaviour will be referred to later.

In order to study the effects of the acetate and the glucose present in the first experiment separately, two experiments were carried out in which either the

acetate or the glucose was left out. The results of these experiments are collected together in Figs. 2 and 3.

A comparison of Figs. 2 and 3 with the graph in Fig. 1 shows that the decomposition of glucose in the second experiment, in spite of the absence of acetate,

Fig. 2.

Fig. 3.

Fig. 2 (Exp. 2). Pope medium with 0.2% glucose, without acetate. A, Glucose; B, pH; C, 1/Lf.

Fig. 3 (Exp. 3). Pope medium with acetate, without glucose. A, pH; B, 1/Lf.

takes place with practically the same velocity as in the first experiment. In this case again, the formation of toxin commenced before the glucose had completely disappeared from the solution, but in Exp. 2, and to a more marked degree in Exp. 3, the amount formed was considerably less than in Exp. 1. The pH curve in Exp. 2 is similar in form to that in Exp. 1. In Exp. 3, the small decrease in pH at the beginning is probably due to the removal of traces of carbohydrates, which are present in the usual brands of peptone and which cause the formation of small amounts of acid by their decomposition.

To complete this series Exp. 4 was carried out in which the additions of both acetate and glucose were omitted. The result is given in Fig. 4.

As regards the formation of toxin, this is again much less than in any of the previous experiments. With regard to the course of pH, especially the small decrease at the beginning of the experiment, the same remarks apply as in Exp. 3.

Since it appeared from various preliminary experiments which we carried out, in agreement with most of the data in the literature, that the optimum concentration of glucose was 0.2%, it was of interest to try the effect of increasing the amount of glucose. Exp. 5 was therefore carried out with 0.5% glucose (Fig. 5).

Although the diphtheria bacteria are able eventually to decompose this relatively large amount of glucose, the pH falls so much that, when all the glucose has disappeared from the solution, the conditions are too unfavourable for further development, so that there is no longer a question of pH reversal. Toxin is formed only in very small quantities and, as the high degree of acidity indicates, is destroyed as the experiment continues; at least it can no longer be determined by the flocculation test.

In recent years, the use of maltose instead of glucose in diphtheria culture media has been stressed more and more. In general, better results are obtained than was previously the case with glucose. Ström and others are of the opinion that the reason for this is that the maltose will be slowly converted into glucose, so that the diphtheria bacteria have ample opportunity for acting further on the decomposition products of glucose, whilst a smaller decrease in pH occurs. At the same time the growing culture is in contact with a small amount of glucose for a longer time.

Since preliminary experiments had indicated that the diphtheria bacteria could tolerate a concentration of 1 % of maltose in the Pope medium, Exps. 6 and

Fig. 4.

Fig. 5.

Fig. 4 (Exp. 4). Pope medium without acetate and without glucose. A, pH ; B, $1/Lf$.

Fig. 5 (Exp. 5). Pope medium with acetate and 0.5 % glucose. A, Glucose; B, pH ; C, $1/Lf$.

7 were carried out in which 1.0 % and 0.3 % respectively of maltose were added to the medium. This latter concentration was chosen in connexion with a routine medium to which a similar quantity of maltose is added (see Figs. 6 and 7).

The maltose used was, with one exception (Exp. 6), supplied by the firm of Hoffmann La Roche & Co., Basel, and had the following characteristics: colour: white; solubility in water (1:1): complete; reaction to litmus: neutral; ash content: trace; $[\alpha]_D^{20}$ ($c = 5.0$ % in water): 128.4° (lit. 128.5°). No glucose could be detected in a 10 % solution of this sugar by inoculation with a thick suspension of *Torula monosa* [Kluyver, 1914].

From a consideration of the figures, it follows directly that maltose is decomposed only very slowly and also that it never disappears completely from solution. The reason for the latter phenomenon must be left undecided for the moment. The pH decreases relatively little and then slowly rises. More remarkable in this connexion is the second rise in pH , which occurs after about 220 hours in Exp. 6. We have established that this is not fortuitous by various control experiments. If 1 % of maltose is added, the concentration falls almost linearly over a considerable period of time until, after about 170 hours, the fall becomes slower. The addition of maltose instead of glucose to the culture medium greatly favours the formation of toxin. This is most striking in Exp. 7 with 0.3 % of maltose. Further, it should be noted here, that the experiments with 0.3 % of

maltose gave a more regular and consistent production of toxin than those with 1 % of this sugar.

Since the diphtheria bacterium is able on the one hand to decompose not more than 0.2 % of glucose without detriment to the formation of toxin, yet on the other hand can decompose 1 % of maltose quite smoothly, the question

Fig. 6 (Exp. 6). Pope medium with acetate and 1 % maltose. A, maltose; B, pH; C, 1/Lf.

Fig. 7 (Exp. 7). Pope medium with acetate and 0.3 % maltose. A, maltose; B, pH; C, 1/Lf.

arises as to what the power of decomposing so much more maltose must be ascribed. Ström's opinion on this question has already been stated (p. 1760).

On the grounds of the ratio 0.2 % glucose:1 % maltose, it may be supposed that the decomposition of glucose can proceed five times as rapidly as its formation from the mother substance, maltose. It appeared to be of interest to test this hypothesis experimentally by measuring the ratio of the two velocities more accurately. For this purpose Exp. 8 was carried out. To a stock solution containing acetate, 0.2 % of glucose was added to one half and 0.2 % of maltose to the other. Each solution was then divided between two flasks (each of two litres

capacity). At definite times the sugar content was determined in all four flasks; in these experiments the pH and $\frac{1}{L_f}$ determinations were omitted. The results are given in Fig. 8, with regard to which it should be mentioned that the relative concentrations of maltose and glucose shown are the means of the separate values in the duplicate experiments.

The ratio of the velocities of the decomposition of glucose and the hydrolytic fission of maltose was now derived graphically as follows. The decomposition of glucose takes place during the interval of time between 15 and 35 hours after inoculation (i.e. the steepest part of the curve) with practically constant velocity. Thus this occurs between the points A and B. During this period, the relative concentration of glucose falls from 95% to 12%, i.e. by 83%. During the same interval of time, the concentration of maltose falls from 94% to 81%, i.e. by 13% between the points C and D. Thus roughly, the ratio of the velocities can be calculated as $83/13 = \text{about } 6$. In other words, the amount of glucose formed by the hydrolytic fission of maltose is decomposed six times more rapidly than it is formed from maltose. This result agrees very well with the ratio figure ≥ 5 obtained above from very superficial considerations.

Fig. 8.

Fig. 9.

Fig. 8 (Exp. 8). Pope medium with acetate and 0.2% glucose, or 0.2% maltose. A, Glucose; B, maltose.

Fig. 9 (Exp. 9). Pope medium with acetate and 0.2% glucose. Proteose-peptone substituted by Witte peptone. A, Glucose; B, pH ; C, $1/L_f$.

On the grounds of these facts we should not expect to be able to detect glucose along with maltose during the incubation of substrates to which maltose has been added.

We repeated an experiment with the Pope medium containing 1% of maltose besides acetate. Samples of 500 ml. were taken after 9, 21 and 312 hours' incubation and each evaporated on the water-bath to ten times the initial concentration. The latter solutions contained 9.17, 8.60 and 1.76% of sugars, respectively, calculated as maltose. Dr L. H. C. Perquin very kindly tried under the supervision of Prof. A. J. Kluver to determine biochemically any glucose present along with the maltose but was unable to do so. To both these gentlemen we wish to take this opportunity of expressing our heartiest thanks for their collaboration.

So far, these substrates have all contained the same source of nitrogen, namely, Difco-proteose-peptone. It was important to try other sources of nitrogen in this investigation. In the first place (Exp. 9, Fig. 9), we chose the well-known brand of Witte peptone of Rostock for this purpose. The media prepared for these experiments had exactly the same composition as the Pope medium, except that 2% of proteose-peptone was replaced by 2% of Witte peptone.

The poor growth and small film formation were striking. This brand of peptone, although fairly generally used previously for the preparation of diphtheria culture media, is very poorly suited for use as the sole source of nitrogen and stands in this respect very far behind Difco-proteose-peptone. Quantitatively this shows itself in a considerably reduced and very slow decomposition of sugar. Also the pH falls slowly and shows only a very feeble reversal. The formation of toxin is extremely small, but it is worth noting that, even in this experiment, the formation of toxin begins before the glucose has completely disappeared from the solution.

Finally two other culture media were examined in this investigation (Exps. 10 and 11, Tables I and II), which for convenience will be called by the names under which they are known in our Institute. The Tomcsik medium is prepared chiefly by the well-known recipe of Martin, meat extract + peptone from pig's stomach with the addition of 0.5% of sodium acetate and 0.2% of glucose, pH 7.6, whilst the Bandoeng medium differs from the one just described only in a few minor particulars, except that in place of 0.2% of glucose, 0.3% of maltose is added, pH 8. Since these results give graphs, which are almost identical with those in Figs. 1 and 7, the latter may be referred to in this case.

It appears that these experiments agree very closely with the corresponding experiments using the original Pope medium and 0.2% of glucose and 0.3% of maltose, respectively (see Figs. 1 and 7), and therefore call for no further comment.

A summarized version of the results is given in Table III.

DISCUSSION.

It appears that the diphtheria bacteria are able to decompose glucose completely at a maximum concentration of 0.5%, whilst maltose never disappears completely from the substrate. It is difficult to see the reason for this phenomenon. In spite of satisfactory growth at the beginning and during the first few days of the experiment, at a certain time a retardation or cessation of metabolism occurs, and the sugar still present is decomposed no further. Possibly factors connected with the nitrogen metabolism play a part here. However, until more data on this subject are forthcoming, there is little more to say.

As regards the velocity of decomposition of glucose, this appears to be practically independent of whether or not acetate is present, but depends far more on the nature of the source of nitrogen present. In this connexion, a combination of meat extract + pig's stomach peptone as used in the Tomcsik medium, is practically of equal value to the Difco-proteose-peptone used in the Pope medium. Witte peptone, however, which gives a very poor growth and so brings about a very slow decomposition of the sugar, behaves quite oppositely. The fact that the sugar in a Pope medium with 0.5% of glucose is decomposed rapidly at first but later is completely used up only slowly, is probably due entirely to the high degree of acidity developed in the later stages of the experiment.

Considering now the alteration in the hydrogen ion concentration in the various experiments, the following statement may be made. The pH falls more or less considerably in all cases where glucose is decomposed by the bacteria either

Table I. *Tomcsik medium with 0.5% sodium acetate and 0.2% glucose.*

Hours of cultivation	pH	Glucose		$\frac{1}{Lf}$
		Absolute quantities in %	% of initial quantities	
0	7.46	0.199	100	—
8	7.46	0.199	100	—
16	7.40	0.199	100	—
24	7.18	0.179	90	—
28	6.95	0.164	82	—
32	6.57	0.141	71	—
36	6.23	0.100	50	—
40	5.88	0.057	29	1.0
50	6.55	—	—	6.5
62	7.61	—	—	10
73	8.25	—	—	13
86	8.35	—	—	13
97	8.47	—	—	13
121	8.76	—	—	13
145	8.92	—	—	13

Table II. *Bandoeng medium with 0.5% sodium acetate and 0.3% maltose.*

Hours of cultivation	pH	Maltose		$\frac{1}{Lf}$
		Absolute quantities in %	% of initial quantities	
0	7.94	0.276	100	—
12	—	0.276	100	—
24	7.88	0.276	100	—
48	7.63	0.261	95	—
60	7.56	0.229	83	1.0
72	7.85	0.190	68	—
74	—	—	—	7.8
79	7.99	0.174	63	9
84	8.04	0.169	61	—
96	8.14	0.145	53	—
120	8.49	—	—	—
123	—	—	—	18
145	8.64	0.112	41	19
169	8.87	0.105	38	19
216	9.02	0.101	33	19
266	9.16	0.101	33	19

Table III. *Summary of velocities of sugar breakdown in the various culture media.*

Medium	Specimen of peptone	Presence of acetate	Sugar used	Time at which the sugar has disappeared hours
Pope	Difco-proteose	+	0.2% glucose	32
	"	—	0.2% glucose	34
	"	+	0.5% glucose	120
	"	+	1% maltose	∞
	"	+	0.3% maltose	∞
	Witte	+	0.2% glucose	98
Tomcsik	Pig's-stomach	+	0.2% glucose	43
Bandoeng	Pig's stomach	+	0.3% maltose	∞

directly or after formation from maltose, doubtless owing to the production of various acids by the degradation of the glucose. The fall in pH is greater, the greater the amount of glucose available for decomposition. In experiments using 0.2% of glucose, the lowest pH observed was 5.88 in the case of the Tomcsik medium, whilst the experiment with 0.5% of glucose showed a fall to 5.15. It is of course obvious that this lowest pH value and the course of the pH curve are dependent on the buffer capacity of the medium employed (the kind of peptone used, the presence or absence of acetate etc.).

What causes this rise in pH , which always occurs in cases that are physiologically normal for the bacteria in question? The compilers of the monograph "Diphtheria", already mentioned, give it as their opinion on this point, that after the acids have reached a certain concentration, the bacteria oxidize them to CO_2 (and H_2O), which combines with the alkali originally present to form carbonate and bicarbonate and so explains the inflexion of the pH curve. Although an experimental proof of this hypothesis is still wanting, a fact which strongly supports it is, that in all cases of direct decomposition of glucose, the minimum pH coincides for all practical purposes with the disappearance of the sugar from the medium. One must suppose therefore that glucose, as such, is an easily available source of energy of great value, which is first assimilated practically completely, before the less readily accessible, poorer source of energy, the acids, is consumed. The facts that the pH in the experiments with maltose decreased less and that the minimum in the pH curve was less well defined, is also in agreement with the above. In these cases of indirect glucose decomposition, both processes occur simultaneously, splitting of glucose into acids and oxidation of the latter to carbonates. Finally, in this connexion, an attempt must be made to find an explanation for the second rise in the pH curve observed in experiments with 1% of maltose. In this case also, the explanation must be considered hypothetical. When this second rise occurs (after about 220 hours, see Fig. 6), the decomposition of sugar and the formation of toxin are practically at a standstill. Thus, at this time, presumably, there will be no more acid formed in the solution, which may cause a rise in pH by subsequent oxidation to carbonate. The idea suggests itself that this second rise in pH might be the result of the formation of extra ammonia from the peptone present.

The formation of toxin can be considered under three headings: (a) the time at which toxin formation begins, (b) the velocity with which formation takes place, (c) the maximum value reached.

As regards the first point, experiments with glucose give the simplest picture. In all these experiments toxin formation occurs at the time when the glucose practically disappears from the solution. The time at which toxin formation begins (which can only be approximately determined by graphical methods) is given in Table IV, along with the corresponding maximum values.

The figures indicate clearly that this setting off point, both in experiments with maltose and glucose, coincides with the minimum pH .

The question now arises whether anything can be deduced from these phenomena regarding the nature of the toxin formation process. Without going into further details, as experimental data on this point are still wanting, we should like to consider the formation of toxin chiefly as a result of dissimilatory nitrogen metabolism, which begins at the moment when the carbon sources are mostly used up.

In those cases where toxin is formed in reasonable amounts, the velocity of the process is almost constant until the maximum is reached, when it suddenly

Table IV. *Summary of toxin production in the various media.*

Medium	Beginning of toxin-pro- duction after hours	Maximum toxin- value
Pope, proteose-peptone, acetate, 0.2% glucose	30	17
Pope, proteose-peptone, no acetate, 0.2% glucose	28	6.5
Pope, proteose-peptone, acetate, no glucose	30	4.2
Pope, proteose-peptone, no acetate, no glucose	24	1.0
Pope, proteose-peptone, acetate, 0.5% glucose	32	1.3
Pope, proteose-peptone, acetate, 1% maltose	24	26
Pope, proteose-peptone, acetate, 0.3% maltose	31	29
Pope, Witte peptone, acetate, 0.2% glucose	85	1.2
Tomcsik, pig's stomach, acetate, 0.2% glucose	49	13
Bandoeng, pig's stomach, acetate, 0.3% maltose	58	19

falls to zero. The cause of this more or less abrupt cessation in the production of toxin is difficult to diagnose.

This phenomenon may find its origin in the utilization of a necessary constituent for toxin formation, or the attainment of too high a concentration of one or other of the products of metabolism. The absolute value of the velocity (measured by the steepness of the toxin curve) is of course dependent on various factors connected with the metabolism.

As shown by Table IV, the composition of the medium plays a very important part in determining the maximum value obtained in the various experiments for the concentration of toxin. The pronounced effect of the absence or presence of acetate in the medium shows that the decomposition of definite carbon compounds is of great importance for the formation of toxin.

SUMMARY.

1. A series of experiments was carried out with *C. diphtheriae* to determine the velocity of decomposition of the sugar, the changes in the hydrogen ion concentration and the formation of toxin, glucose and maltose being used as sugars, whilst various brands of peptone were employed as sources of nitrogen.

2. The diphtheria bacteria are able to assimilate glucose up to a concentration of 0.2% without detriment to the formation of toxin. If more glucose is added (0.5%), the total amount of sugar is decomposed, but such a large amount of acid is formed that the production of toxin is almost completely inhibited. The decomposition is slower than with the smaller concentration of sugar. On the other hand maltose is tolerated in much higher concentrations. The cause of this phenomenon appears to lie in the fact, that the velocity of decomposition of glucose by diphtheria bacteria is about six times greater than the velocity of formation of glucose by the hydrolysis of maltose.

3. In the decomposition of the sugar, the pH first of all falls until a point is reached when the sugar (glucose) disappears from the solution, and then it rises again, owing probably to oxidation of the acids, formed initially from the glucose, to CO₂ and H₂O, which combines with the alkali originally present to basically reacting carbonate and bicarbonate.

4. The formation of the toxin proper begins at the moment when the sugar (glucose) practically disappears from the solution, continuing during a definite period with practically constant velocity, to cease quite suddenly. The $\frac{1}{L}$ value, measured by means of Ramon's flocculation test, does not decrease on continued incubation of the culture.

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