A STABILIZING EFFECT OF HEMOGLOBIN ON THE PNEUMOCOCCUS TRANSFORMATION

YUKITO MASAMUNE, MIHOKO ABE AND DEN'ICHI MIZUNO

Department of Chemistry, National Institute of Health, Tokyo

(Received: April 15th, 1960)

In experiments of Pneumococcus transformation, several media have been devised in an attempt to improve the reproducibility of results ever since the *in vitro* reaction was first carried out by Dawson (1931). It has been recognized that the occurrence of the *in vitro* reaction requires some environmental factors, in addition to the nutrients necessary for the initiation and maintenance of cell growth (McCarty *et al.*, 1946). Rabbit serum or human serous fluids provided such factors in many cases (E-Taylor, 1949; Austrian, 1952). Hotchkiss and E-Taylor (1951) pointed out that bovine albumin could substitute the factors. However even in the presence of these factors, a reproducible and stable result was not necessarily secured in the Pneumococcus transformation. In order to obtain a constant and regular result in this reaction, it will be necessary to supply a better qualified medium and appropriate factor(s). At the same time, elucidation of the nature of such factor(s), if any, will be significant to an understanding of the mechanism of the transformation reaction.

During the course of experiments performed in our laboratory on Pneumococcus transformation from SM sensitive to resistant cells, using heart infusion Neopepton broth as a basic medium, it was found that an addition of albumin and of horse blood hemolysate gave good results, while irregular results were obtained with the addition of albumin only. The experiments performed to ascertain the nature of the substance(s) in the hemolysate will be presented below.

MATERIALS AND METHODS

Strains: Diplococcus pneumoniae, RSt5b (SM resistant, DNA donor strain) and R19 (recipient strain) were the same as in the previous report (Abe and Mizuno, 1959).

Preparation of DNA from the SM resistant strain, and transformation reaction: The same technique as described before was followed (Abe and Mizuno, 1959). The assay of transformants was always done in agar plates containing SM (150 µg/cc) 100 minutes after the addition of DNA.

Medium: The basic medium contained per liter Heart infusion broth 'Difco' 10g, Neopepton 'Difco' 10g and Na₂HPO₄·2H₂O 2.5g, and was brought to pH 7.6-7.8 with a NaOH solution. Nutrient broth for cultivation of DNA donor cells, solid medium for the counting of SM resistant transformants and of survival cells were the same as described previously (Abe and Mizuno, 1959). Bovine albumin (Armour Lab.) was added to the basic medium for the transformation reaction to make the final concentration of 0.2%. This is called '*transformation medium*'.

* Aided in part by a grant of the Hôan-sha Foundation.

** The following abbreviations will be used: Streptomycin (SM); Deoxyribonucleic acid (DNA); Brom phenol blue (BPB).

正宗行人・阿部美穂子・水野伝一 (国立予防衛生研究所化学部)

Preparation of hemolysate: Fresh defibrinated horse blood was centrifuged, and the blood cells precipitated were added [with distilled water to 2 times the original volume of blood. The lysed cells were centrifuged at 3,000 rpm for 10 min. The supernatant will be called '*hemolysate*'. The hemolysate was kept frozen and added to the medium prior to use. The concentration of the *hemolysate* in the medium will be shown in v/v %.

Preparation of hemin, oxyhemoglobin and methemoglobin: Hemin was prepared from horse blood as described by Akabori (1949). Oxyhemoglobin and methemoglobin were prepared by the following method (Egami *et al.*, 1953).

Oxyhemoglobin: Fresh horse blood cells were washed several times with saline, and added with an equal volume of distilled water, which was followed by an addition of half a volume of ether. After centrifugation of the lysed mixture, the water layer containing hemoglobin was dialysed in a cellophan bag against distilled water. The hemoglobin was crystallized within the bag. It was collected and dissolved in M/15 Na_2HPO_4 solution, and its extinction at 575 m μ was adjusted to that of *hemolysate*. This preparation was used as '*crystalline oxyhemoglobin*'.

Methemoglobin: A few drops of 5% ferricyanide solution were added to the *crystalline oxyhemoglobin* solution to convert it to methemoglobin. The excess ferricyanide was dialysed off against water. The dialysed solution was used as '*methemoglobin*'.

RESULTS

Effect of the Hemolysate Added to the Basic Medium

The basic medium mentioned above could not support the growth of cells without treatment by charcoal or addition of serum or of albumin. The charcoal treatment (Tolmach, 1957) of the medium was not adopted to obtain satisfactory, reproducible results, because of the difficulty of regulating the conditions. As shown below the cells grown in the *transformation medium* were competent to the reaction, though within a limited period and at a low rate. We added the *hemolysate* to this medium. The *hemolysate* added alone in 0.5 to 1% in the basic medium was able to support the growth of cells but could not support the transformation reaction. The effect of the *hemolysate* added to the *transformation medium* in 0.01 to 1% is indicated in Table 1. The *hemolysate* in concentrations of 0.1 to 1% promoted the reaction.

Table 1. Effect of the *hemolysate* to support *Pneumococcus* transformation in the '*transformation medium*'*

	Supplement to the <i>transformation medium</i> *	I Viable cells/cc	II SM resistants/cc	II/I Transformation rate
Exp. 1	None	1.4×10^7	< 10	—
	Hemolysate 1%	2.2×10^7	1.8×10^4	7.9×10^{-4}
	Hemolysate 0.1%	1.5×10^7	9.5×10^3	6.2×10^{-4}
	Hemolysate 0.01%	2.1×10^7	1.0×10^1	4.8×10^{-7}
Exp. 2	None	2.2×10^7	< 10	—
	Hemolysate 1%	2.3×10^7	4.9×10^4	2.1×10^{-3}
	Hemolysate 0.1%	2.4×10^7	1.7×10^4	7.0×10^{-4}
	Hemolysate 0.01%	1.9×10^7	< 10	—

* The *transformation medium* is the basic medium added with 0.2% albumin.

The basic medium contained heart infusion 'Difco' 10 g, Neopepton 'Difco' 10 g and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 2.5 g in 1 l and was adjusted to pH 7.6–7.8 with NaOH.

Table 2. Effect of the supernatant* of the *hemolysate* to support *Pneumococcus* transformation in the *transformation medium***

	Supplement to the <i>transformation medium</i> **	I Viable cells/cc	II SM resistants/cc	II/I Transformation rate
Exp. 1	None	9.8×10^6	< 10	—
	Hemolysate 1%	1.2×10^7	6.0×10^4	5.1×10^{-3}
	Supernatant 1%	9.1×10^6	5.3×10^4	5.8×10^{-3}
Exp. 2	None	1.2×10^7	1.2×10^4	1.0×10^{-3}
	Hemolysate 1%	1.6×10^7	2.6×10^5	1.6×10^{-2}
	Supernatant 1%	2.3×10^7	2.4×10^5	1.1×10^{-2}

* The supernatant was obtained by centrifugation of the *hemolysate* at 100,000 g, for 40 min.

** See Table 1.

Ultracentrifuge Fractionation of the Hemolysate

In order to know what component(s) of the *hemolysate* would show the promoting effect on the transformation reaction, the *hemolysate* was fractionated by ultracentrifugation. The *hemolysate* was centrifuged at 100,000 g for 40 minutes to isolate a supernatant from precipitable particles. The activity of the supernatant, as is presented in Table 2, was almost comparable to that of control (*hemolysate*), showing the presence of the active principle in the soluble fraction.

Dialysis of the Hemolysate

The effect of the dialysed *hemolysate* is shown in Table 3. The *hemolysate* was dialysed in a cellophan bag against distilled water in a cold-room for 24 hours. The

Table 3. Effect of the dialysed *hemolysate** to support *Pneumococcus* transformation in the *transformation medium***

	Supplement to the <i>transformation medium</i> **	I Viable cells/cc	II SM resistants/cc	II/I Transformation rate
Exp. 1	None	3.3×10^6	3.0×10^3	9.1×10^{-6}
	Hemolysate 1%	5.3×10^6	1.9×10^5	3.6×10^{-2}
	Dialysed hemolysate 1%	6.9×10^6	4.4×10^4	6.4×10^{-3}
Exp. 2	None	4.8×10^6	3.0×10^3	6.7×10^{-4}
	Hemolysate 1%	8.9×10^6	9.9×10^4	1.1×10^{-2}
	Dialysed hemolysate 1%	1.1×10^7	8.3×10^4	7.6×10^{-3}
Exp. 3	None	2.7×10^6	6.0×10^2	2.2×10^{-4}
	Hemolysate 1%	4.6×10^6	6.0×10^3	1.3×10^{-3}
	Dialysed hemolysate 1%	6.0×10^6	8.0×10^3	1.3×10^{-3}

* The *hemolysate* was dialysed in a cellophan bag against distilled water in a cold room for 24 hrs.

** See Table 1.

Table 4. Effect of the dialysable fraction of hemolysate to support *Pneumococcus* transformation in the *transformation medium***

	Supplement to the <i>transformation medium</i> **	I Viable cells/cc	II SM resistants/cc	II/I Transformation rate
Exp. 1	a. None	2.1×10^7	< 10	—
	b. Hemolysate 1%	2.0×10^7	2.3×10^4	1.2×10^{-3}
	c. Dialysed hemolysate 1%	2.2×10^7	1.2×10^3	5.5×10^{-4}
	d. Dialysable fraction 1%	2.1×10^7	8.0×10^1	3.8×10^{-6}
	e. c plus d 1% each	2.6×10^7	2.6×10^3	1.0×10^{-3}
Exp. 2	a. None	9.8×10^6	< 10	—
	b. Hemolysate 1%	1.2×10^7	6.0×10^4	5.1×10^{-3}
	c. Dialysed hemolysate 1%	1.1×10^7	1.8×10^4	1.7×10^{-3}
	d. Dialysable fraction 1%	1.1×10^7	1.4×10^2	1.3×10^{-5}
	e. c plus d 1% each	1.3×10^7	2.8×10^4	2.2×10^{-3}
	f. Oxyhemoglobin 1%	1.3×10^7	1.4×10^4	1.0×10^{-3}
	g. f plus d 1% each	1.1×10^7	5.3×10^4	4.9×10^{-3}

* The dialysed fraction was evaporated to the initial volume of the *hemolysate* by a flushing evaporator at *ca.* 35°C.

** See Table 1.

Table 5. F-test of mean values of transformation rates in the medium supplemented with the hemolysate fractions

Supplement to the <i>transformation medium</i> *	Transformation rate ($\frac{\text{SM resistants}}{\text{viable cells}}$)					Mean $\bar{X}_i$	Variance S_i^2
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5		
a. Hemolysate	1.4×10^{-8}	1.3×10^{-8}	2.7×10^{-8}	1.6×10^{-8}	1.6×10^{-8}	1.7×10^{-8}	0.25×10^{-6}
b. Dialysed hemolysate	$< 10^{-8}$	2.7×10^{-8}	0.55×10^{-8}	1.9×10^{-8}	0.4×10^{-8}	1.1×10^{-8}	1.04×10^{-6}
c. b plus dialysable fraction	2.9×10^{-8}	1.5×10^{-8}	2.2×10^{-8}	1.6×10^{-8}	1.1×10^{-8}	1.9×10^{-8}	0.40×10^{-6}

The differences among the variances (S_i^2) of (a) (b) (c) have no significance at 5% level. The mean values ($\bar{X}_i$) of them also have no significant differences at 5% level.

* See Table 1.

dialysed *hemolysate* was added to the *transformation medium* in the concentration of 1%. Although the dialysed *hemolysate* showed a lower activity than the original *hemolysate* in most cases, a significant promotive effect of the dialysed *hemolysate* was recognized as compared with that exerted by the addition of albumin only.

The dialysable fraction was evaporated to the initial volume of the *hemolysate* by a flushing evaporator at about 35°C. The effect of this solution which was added to the *transformation medium* in the concentration of 1% is indicated in Table 4. The dialysable fraction had little effect when added alone to the *transformation medium*. But when added together with the dialysed hemolysate, the results seemed to suggest

the presence of some factor(s) in the dialysable fraction which could restore, to some extent, the lowered activity of the non-dialysable fraction (Table 4). Therefore, we tried to analyse statistically the significance of the differences between the transformation rate obtained by the medium with the non-dialysable fraction only and that with both the dialysable and non-dialysable fractions. The results showed that we could not expect the presence of any factor(s) in the dialysable fraction which might have an additive effect (Table 5).

Heat Stability

Fig. 1 represents the effect of heat on the activity of the *hemolysate* to support the transformation reaction. The *hemolysate* was heated at each temperature indicated for

Fig. 1. Heat stability of the *hemolysate* and the *oxyhemoglobin*.

The hemolysate (O) or the oxyhemoglobin (X) was heated at each temperature indicated for 1 hour. The activity was expressed by transformation rate $\left(\frac{\text{SM resistant/cc}}{\text{viable cells/cc}} \right)$.

The ordinate indicates the activity of the heated sample as percentage of the original hemolysate; the abscissa indicates the temperature where the sample was heated.

60 minutes, and each heated hemolysate was added in the same concentration as that of the non-heated hemolysate. The curve of inactivation of the *hemolysate* dropped at around 60°C. The activity was lost completely at 100°C. Hemin was prepared from horse blood and added to the medium, but it did not reveal any activity to support the transformation reaction.

Electrophoretic Separation of the Dialysed Hemolysate

The dialysed hemolysate was separated electrophoretically in the following conditions: M/15 phosphate buffer, pH 8.0, Whateman No.1 filter paper, strip 4 cm × 30 cm, 0.3 mA per cm², 300 V, for 23 hours, in a cold room. The paper was stained with BPB. As is illustrated in Fig. 2, a densitometrically single peak was obtained.

Fig. 2. Electrophoretic protein pattern of the dialysed *hemolysate*.

Effect of Hemoglobin

It seems likely, from the results described above, that the active component of the *hemolysate* is a soluble protein, probably oxyhemoglobin which is a principal component of erythrocyte. The effect of the *crystalline oxyhemoglobin* to support the transformation reaction is presented in Table 6. The *crystalline oxyhemoglobin* showed, sometimes, a lower activity than the control *hemolysate*, but it was definitely promotive to the reaction as compared with the control employing the *transformation medium* only. The heat stability of the *oxyhemoglobin* was the same as that of the *hemolysate*. Therefore, it seems likely that the effect of the non-dialysable fraction depends essentially on the presence of oxyhemoglobin therein.

Effect of Methemoglobin

A question arose as to whether or not the hemoglobin had to be an oxygenated form *i.e.* oxyhemoglobin, to play an essential role in the transformation reaction.

Methemoglobin was tested in comparison with oxyhemoglobin. The results are cited in Table 7. The methemoglobin showed a lesser effect than oxyhemoglobin in a comparable concentration.

Appearance and Maintenance of Cell Competency in the Presence of Supplements for the Basic Medium

For the comparison of the effect of supplements on the basic medium, the following

Table 6. Effect of the *crystalline oxyhemoglobin** to support *Pneumococcus* transformation in the *transformation medium***

	Supplement to the <i>transformation medium</i> **	I Viable cells/cc	II SM resistants/cc	II/I Transformation rate
Exp. 1	None	4.8×10^6	3.0×10^3	6.7×10^{-4}
	Hemolysate 1%	8.9×10^6	9.9×10^4	1.1×10^{-2}
	Crystalline oxy- hemoglobin 1%***	1.4×10^7	7.0×10^4	5.1×10^{-3}
	Dialysed hemolysate 1%	1.1×10^7	8.3×10^4	7.6×10^{-3}
Exp. 2	None	3.3×10^6	3.0×10^1	9.1×10^{-6}
	Hemolysate	9.8×10^6	9.2×10^4	9.5×10^{-3}
	Crystalline oxy- hemoglobin 1%	1.1×10^7	3.9×10^3	4.5×10^{-3}
	Dialysed hemolysate 1%	1.4×10^7	4.4×10^3	3.2×10^{-3}

* The hemoglobin was crystallized from horse blood cells.

** See Table 1.

*** The concentration of hemoglobin was adjusted by the extinction at $575 \mu\mu$, to that of the *hemolysate*. This preparation was called *crystalline oxyhemoglobin*.

Table 7. Effect of the *methemoglobin** to support *Pneumococcus* transformation in the *transformation medium***

	Supplement to the <i>transformation medium</i> **	I Viable cells/cc	II SM resistants/cc	II/I Transformation rate
Exp. 1	a. Hemolysate 1%	1.6×10^7	6.8×10^4	4.3×10^{-3}
	b. Methemoglobin 1%	1.5×10^7	1.4×10^2	9.7×10^{-4}
	c. Dialysable fraction 1%	1.4×10^7	< 10	—
	d. b plus c, 1% each	2.0×10^7	3.4×10^4	1.7×10^{-3}
Exp. 2	a. Hemolysate 1%	2.2×10^7	1.8×10^4	8.1×10^{-4}
	b. Methemoglobin 1%	2.2×10^7	3.0×10^2	1.4×10^{-5}
	c. Dialysable fraction 1%	1.9×10^7	< 10	—
	d. b plus c, 1% each	2.4×10^7	8.6×10^3	3.6×10^{-4}

* Methemoglobin was prepared by oxydation of oxyhemoglobin with ferricyanide.

** See Table 1.

was used as such: albumin 0.2%; albumin 0.2% and *hemolysate* 1%; albumin 0.2% and *crystalline oxyhemoglobin* 1%. Each medium containing either one of the supplements was inoculated with an equal number of preincubated cells, and the cells grown in each medium were tested for their competency to the transformation reaction at the time interval indicated. The total survival cell number was also counted. The results are recorded in Fig. 3. It was indicated that the effect to support the growth of cells, expressed by total survival cell count, was the same among all the three supplements.

The cell competency was almost equal in the medium supplemented with either the *hemolysate* or *hemoglobin*. The cell competency in both the media reached the maximum level at around 4 hours after the inoculation, and remained on that level even 6 hours after the inoculation. On the other hand, in the medium added with albumin only, the cells showed a considerably high competency at an early phase, though it was lower than in the case of albumin plus the *hemolysate*. However, the competency in this case did not last so long and eventually was lost rapidly. This fact may account for the irregularity of the transformation rate in the medium supplemented with albumin only, as experienced so far in many experiments.

Fig. 3. Cell competency in the culture grown in the basic medium containing supplements.

The basic medium (see Table 1) was supplemented with albumin 0.2% (Δ), albumin and the *hemolysate* ($\circ$) or albumin and *hemoglobin* ($\times$). Total survival cell count: -----, Transformant count: ———.

DISCUSSION

The necessity of serous factor in the transformation of various bacterial species and the effectiveness of serum albumin on the *Pneumococcus* transformation have been recognized. In our case, the basic medium containing heart infusion, Neopepton and phosphate could not support good growth of this organism. An addition of albumin or treatment by charcoal was sufficient to grow cells but was not able to support the transformation reaction. These treatments may behave in favor of removing some inhibitory substance(s), probably some free fatty acids (Pollock, 1949) contained in the medium. Besides, a

specific role of albumin to support the transformation reaction was also shown in our case. An additive and, probably, stabilizing effect of the *hemolysate* or oxyhemoglobin added to the basic medium containing albumin was evident in the above experiment. It is very profitable for the study of this reaction that the cell competency to transformation reaction can be kept constant during a broad growth phase in the medium supplemented with the *hemolysate* or with the *oxyhemoglobin* (Fig. 3). It is uncertain whether hemoglobin (or *hemolysate*) is an additive sensitizer to albumin in the transformation reaction, or a substance acting as a factor maintaining the competency of cells. It is of interest to know the mechanism of the specific role of hemoglobin in the medium for the transformation reaction.

It seems likely that the oxygen in the oxyhemoglobin preparation may play a minor part in the activity of oxyhemoglobin, since we could not get any good results when we aerated the culture before the DNA treatment.

SUMMARY

1. The addition of horse blood hemolysate to the medium for *Pneumococcus* transformation, in addition to albumin, showed a promotive effect in supporting the reaction.
2. The *hemolysate* added to the medium of *Pneumococcus* transformation elongated the time duration of the state where the cells were competent to the transformation as compared with the case in which the basic medium was supplemented with albumin only.
3. It was established that the *hemolysate* could be replaced by the crystalline oxyhemoglobin retaining the effect.

REFERENCES

- ABE, M. & MIZUNO, D. (1959): A contribution to the knowledge of *Pneumococcus* transformation during the period between the incorporation of deoxyribonucleic acid and the appearance of streptomycin resistance. *Biochim. Biophys. Acta*, **32**, 464-469.
- AKABORI, S. ed. (1959): [The preparation method of biochemical reagent.] p. 265. Sogen-sha (text in Japanese).
- AUSTRIAN, R. (1952): Bacterial transformation reaction. *Bact. Rev.*, **16**, 31-50.
- DAWSON, M. H. & SIA, R. H. P. (1931): *In vitro* transformation of *Pneumococcal* types. I. A technique for inducing transformation of pneumococcal types *in vitro*. *J. Exper. Med.*, **54**, 681-699.
- EGAMI, F. *et al.* ed. (1952): [Standard Biochemical Experiment]. pp. 112-120, Bunkyo-do (text in Japanese).
- EPHRUSSI-TAYLOR, H. (1949): Additive effect of certain transforming agents from some variants of *Pneumococcus*. *J. Exper. Med.*, **89**, 399-424.
- HOTCHKISS, R. D. & EPHRUSSI-TAYLOR, H. (1951): Use of serum albumin as source of serum factor in pneumococcal transformation. *Fed. Proc.*, **10**, 200.
- LERMAN, L. S. & TOLMACH, L. J. (1957): Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in *Pneumococcus*. *Biochim. Biophys. Acta*, **26**, 68-82.
- MCCARTY, M. EPHAUSSI-TAYLOR, H. & AVERY, O. T. (1946): Biochemical studies of environmental factors essential in transformation of pneumococcal types. *Cold Spring Harbor Symp. Quant. Biol.*, **XI**, 177-183.
- POLLOCK, M. R. (1949): The effect of long-chain fatty acids on the growth of *Haemophilus pertussis* and other organisms. *Symp. Soc. Exper. Biol.*, **3**, 217-232.