has shown that bursts in isolated cortex are maintained by a reverberating network of similar neurones. The results reported here make it difficult to see how such a net could be responsible for bursts in the intact cortex, for if the cell possesses classical properties, one would expect that local polarization would alter the threshold of the cell to synaptic bombardment from the net, thus changing the frequency of firing within bursts. We have compared the lengths of inter-burst intervals with the duration and number of action potentials in the bursts preceding them and found no correlation. Thus the burst mechanism appears to be independent of the mechanism which generates spike discharges during bursts.

A more detailed account of this work proposing a mathematical model of the signal is in the press. Work is in progress which may lead to a further clarification of the mechanisms involved and of the role of very short intervals.

This work has been carried out as part of the research programme of the National Physical Laboratory, and is published by permission of the Director.

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COUPLING OF CYCLIC CHEMOTHERAPEUTIC COMPOUNDS TO IMMUNE GAMMA-GLOBULINS

By Dr. S. DeCARVALHO, Dr. H. J. RAND and A. LEWIS

Laboratory of Cancer Virology and Immunology, Rand Development Corporation, Cleveland, Ohio

BY virtue of their serological specificity, immune γ-globulins may serve as 'guided missiles' to carry and deliver therapeutic agents to target cells containing the specific antigens. This article deals with some of the chemical problems involved in the coupling of therapeutic agents to γ-globulins.

The following procedure represents an adaptation of the method utilized by Heidelberger[1] to couple β-naphthol 3 : 6 sodium disulphonate (*R*-salt) to crystalline egg albumin, and by Kabat[2,3] to couple the same dye to horse serum albumin. Mathé *et al.* make quick reference to having coupled methotrexate to rabbit hyperimmune γ-globulin by diazotization[4], but no details are given.

The method here described was examined in detail and criteria for the end-points of each step of the coupling were developed.

The following materials were used:

Therapeutic compounds. Methotrexate (courtesy of Dr. J. M. Ruegsegger, Lederle Laboratories, Pearl River, New York) (*A*-methopterin) 4-amino-N^{10}-methyl pteroylglutamic acid (MTX), lot No. 1260–01; uracil mustard (courtesy of Dr. H. Petering, Upjohn Co., Kalamazoo), lot, No. 2358*K* (UM); 5-fluorouracil (courtesy of Dr. G. Zbinden, Hoffmann–La Roche, Inc., Nutley, New Jersey), lot No. 009122 (5-FU); tetracycline ('Achromycin V', Lederle), lot No. 4859–23; 6-mercaptopurine hydrate (Nutritional Biochemical Co., Cleveland, Ohio), lot No. 4554 (6MP); chlorambucil (Nutritional Biochemical Co.), lot No. 9981; 'Thio-Tepa' (Lederle), lot No. 4650–91.

Gamma-globulins. Human γ-globulin from pooled serum (Cutter Laboratories, Berkeley, Calif.), lot No. *G*6083 (HSGG); horse anti-tumour hyperimmune γ-globulin, lot No. 257–29 (ref. 5) (HTUGG); horse anti-leukæmia hyperimmune γ-globulin, lot No. 567–*M*1–21 (ref. 5) (HLKGG).

Even though there was successful coupling with all the therapeutic compounds listed, methotrexate, uracil mustard and 5-FU were the only ones investigated in detail.

The coupling develops through the following three steps: (1) diazotization of benzidine; (2) coupling of the therapeutic compound to diazotized benzidine; (3) coupling of the compound obtained in (2) to γ-globulin.

The temperature of all operations is 7°–8° C. All reagents are added drop by drop with constant stirring.

Step I. In a 100-ml. beaker place 37·0 ml. of benzidine reagent (720·0 mg of analytical grade benzidine in 6·50 ml. of 6 N hydrochloric acid added to 31·50 ml. of distilled water). Cool to 8° C in a bath of ice water oversaturated with sodium chloride. Below 6° C this reagent freezes. Over a period of 2 min add 14·00 ml. of pre-cooled sodium nitrite reagent (1·30 g of sodium nitrite in 32·0 ml. of distilled water). The end-point was determined in the following way. Immediately after each addition of 0·1–

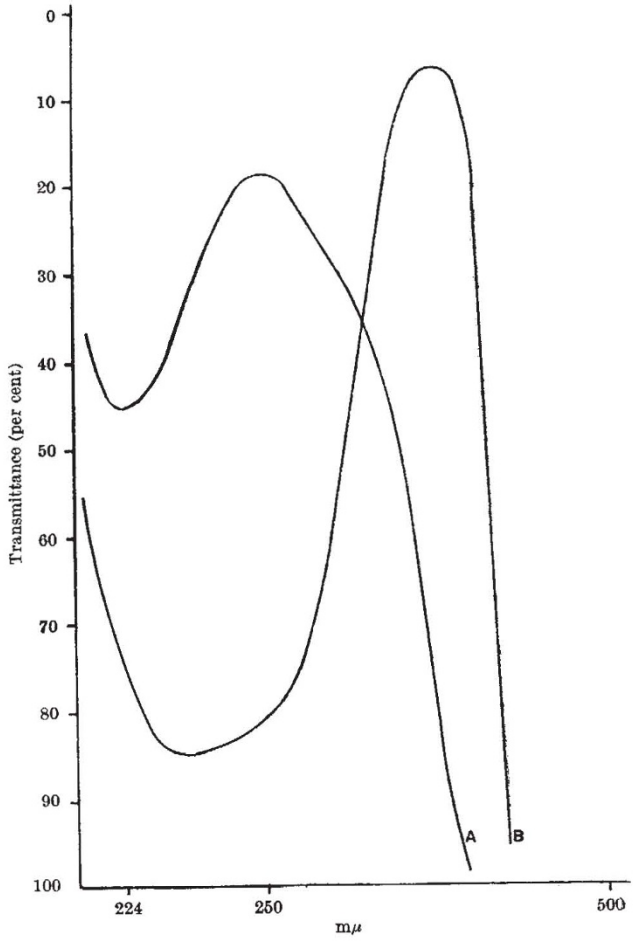


Fig. 1. *A*, 2 μg/ml. benzidine solution in 6 N hydrochloric acid at *p*H 1·0; maximum absorption peak, 250 mμ; minimum absorption peak, 224 mμ. *B*, diazotized benzidine in solution at *p*H 1·0; maximum absorption peak, 313 mμ; minimum absorption peak, 234 mμ

0·2 ml., a piece of starch iodide paper immersed in the solution turns dark blue. This indicates excess of nitrite just added. After stirring for 10 sec, the nitrite is partly combined, partly eliminated and the iodide paper does not discolour. The end-point is indicated by persistence of the blue discoloration of the paper even after stirring for 10–20 sec. However, prolonged stirring (10 min) will again prevent blueing of the paper following volatilization of excess nitrous acid. This product is light green in colour. The colour of the tetrazobenzidine is brought out by addition of sodium acetate (4·50 g of sodium acetate in 10·0 ml. of water). In a 250-ml. beaker place 20·0 ml. of pre-cooled acetate reagent. With constant stirring, add in 1 min the tetrazobenzidine. The colour becomes first light yellowish-green and in 3–5 min changes to yellowish-brown (weak-tea colour). Diazotization of benzidine results in the changes in the ultra-violet absorption spectra indicated in Fig. 1*A* and *B*.

Determination of end points for Steps II and III resulted from the following observations. Keeping the amount of diazobenzidine constant, different amounts of the therapeutic agent were tried to bind to the azobenzidine. It was noticed that at low levels of therapeutic compound the resulting substance when coupled to the γ-globulin produced gelation of the protein while high levels of the cyclic compound prevented gelation.

It was then found that small amounts of tetrazobenzidine alone produced constantly gelation of the γ-globulin.

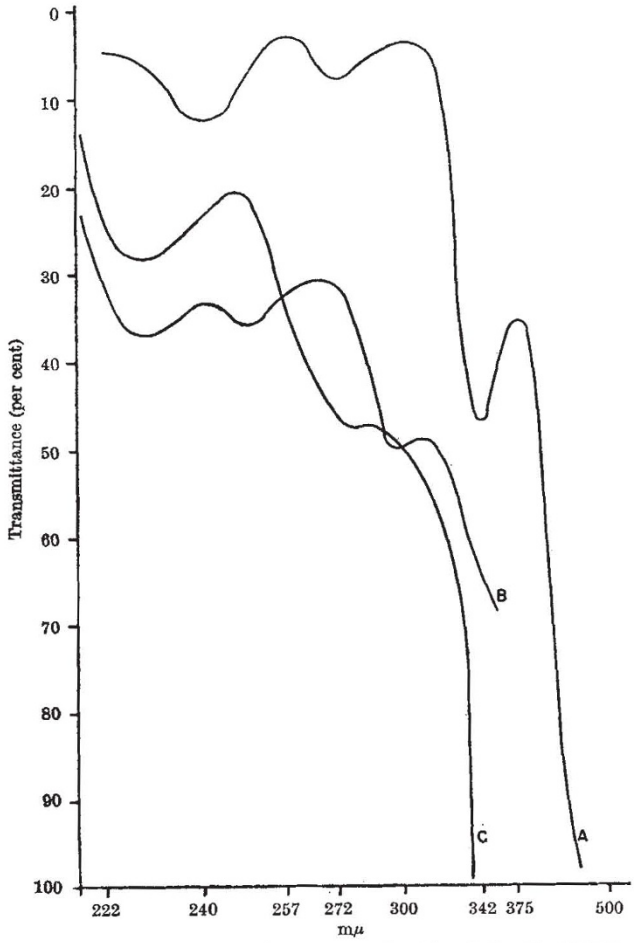


Fig. 2. *A*, 9 μg/ml. solution of methotrexate in sodium hydroxide *p*H 14·0 at 25° C; absorption peaks, maximum, 375 mμ; minimum, 342 mμ; maximum, 300 mμ; minimum, 272 mμ; maximum, 247 mμ; minimum, 240 mμ. *B*, diazo-diphenyl-diazo-methotrexate (azotrexate) solution at *p*H 2·5: absorption peaks, maximum, 369 mμ; minimum, 340 mμ; maximum, 302 mμ; minimum, 271 mμ; maximum, 259 mμ; minimum, 244 mμ. *C*, 1/120 dilution of a γ-globulin-azotrexate ('Gazotrexate-*L*') solution containing 2·4 mg/ml. of methotrexate and 77 mg/ml. of horse anti-leukæmia γ-globulin at *p*H 8·0; absorption peaks, maximum, 369 mμ; minimum, 345 mμ; maximum, 280 mμ; minimum, 250 mμ

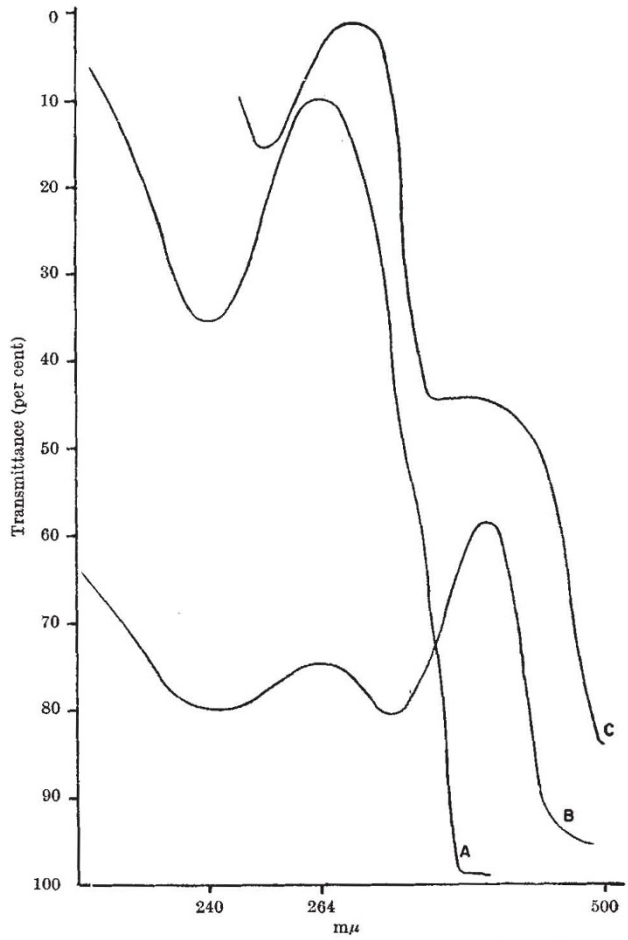
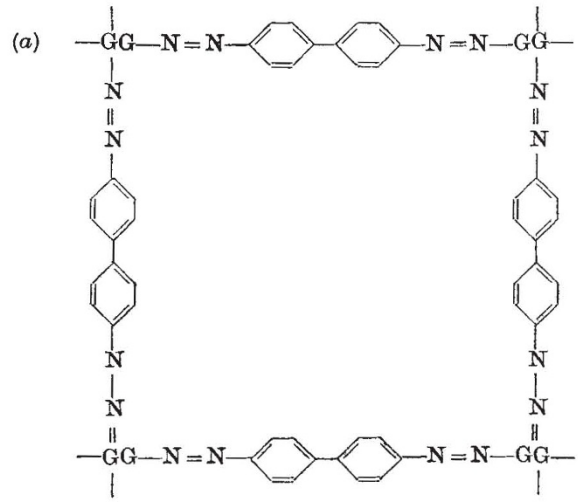


Fig. 3. *A*, 0·00094 per cent solution of 5-FU, *p*H 5·5; maximum absorption peak, 264 mμ; minimum absorption peak, 240 mμ. *B*, solution of diazo-diphenyl-diazo-5-FU (azo-5-FU), *p*H 5·5; absorption peaks, maximum, 340 mμ; minimum, 290 mμ; maximum, 264 mμ; minimum, 240 mμ. *C*, 1/133 dilution of a γ-globulin–azo-5-FU ('Gazo-5-FU–*T*') solution containing: 1·1 mg/ml. of 5-FU and 66 mg/ml. of anti-tumour horse γ-globulin at *p*H 8·0 after dialysis; absorption peaks, maximum, 340 mμ; minimum, 320 mμ; maximum, 280 mμ; minimum, 250 mμ

This led to the hypothesis that tetrazobenzidine acted as a divalent link between the polypeptide chains of γ-globulin (*a*), leading to its copolymerization[7].



On the other hand, the coupling of the tetrazobenzidine to the therapeutic compound (*R*) would either make the azobenzidine non-reactive (*b*) with γ-globulin

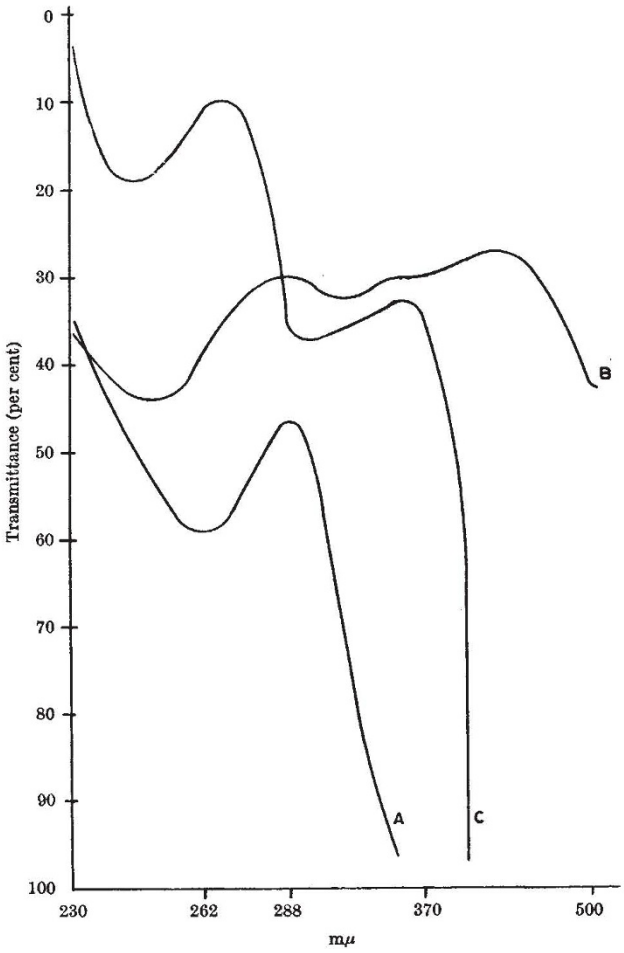


Fig. 4. *A*, 10 μg/ml. solution of uracil mustard, *p*H 11·0; maximum absorption peak, 280 mμ; minimum absorption peak, 262 mμ. *B*, solution of diazo-diphenyl-diazo-uracil mustard (azoum), *p*H 2·5; absorption peaks; maximum, 365 mμ; minimum, 276 mμ; maximum, 252 mμ; minimum, 230 mμ. *C*, 1/20 dilution of a γ-globulin-azoum ('Gazoum *L*') solution containing: 2·0 mg/ml. of UM and 67 mg/ml. of anti-leukæmia horse γ-globulin at *p*H 8·0; absorption peaks, maximum, 390 mμ; minimum, 320 mμ; maximum, 280 mμ; minimum, 250 mμ

(*b*) $R-N=N-\langle\rangle-\langle\rangle-N=N-R$

or univalent (*c*)

(*c*) $R-N=N-\langle\rangle-\langle\rangle-N=N-$

This hypothesis was confirmed by dialysing the final coupled compound extensively against buffered saline and determining the elimination of unbound *R* compound when gelation did not occur. By trial and error the ideal proportions that give a univalent compound (*c*) were determined for each therapeutic agent.

At these proportions no gelation of the protein occurs and only traces of the diazotized agent are found in the buffer outside the dialysing bag.

The following are the proportions found for the three compounds examined. Methotrexate: 600 mg benzidine, 720 mg γ-globulin, 1,920 mg; uracil mustard: 600 mg benzidine, 720 mg γ-globulin, 1,920 mg; 5-fluorouracil: 360 mg benzidine, 720 mg γ-globulin, 1,920 mg.

Step II. To the tetrazobenzidine add the proper amount of the therapeutic agent over a period of 2 min. The mixture slowly acquires a deep brownish-red colour. Allow to stand 7 min at 8° C. To complete coupling add 10·0 ml. of 8 N potassium carbonate. Methotrexate and 5-FU give a strong coffee colour, uracil mustard a red-orange or brick. The solutions are left stationary at 8° C for 1 h in the dark.

Figs. 2*A*, 2*B*, 3*A*, 3*B*, 4*A* and 4*B* give the changes in the ultra-violet absorption spectra following these reactions. These spectra were utilized to identify these compounds in the buffer outside the dialysis bag of the final compound.

Step III. The γ-globulin reagent is prepared by adding to 120 ml. of 16 per cent solution of γ-globulin in isotonic saline 20·0 ml. of a solution containing 11·05 g of potassium carbonate. The protein solution is placed in a 250-ml. beaker and the azotized drug is added slowly. The stirring at this step must be very gentle to avoid denaturation by foaming. At the end, 10·0 ml. of 8 N potassium carbonate is added. The colour of the coupled γ-globulin is essentially the same as that of the tetrazo compound, but the ultra-violet spectrum is specific for each one of them (Figs. 2*C*, 3*C* and 4*C*).

In all the spectra of the Gazo-products the presence of γ-globulin (280/250 mμ) is recognized.

The sequence of the three steps is exemplified for methotrexate in Fig. 5. The exact position of the coupling in the therapeutic compound is not known.

The final product is dialysed against isotonic sodium chloride. The extent of the dialysis is determined by examining the ultra-violet absorption spectra of samples of the saline. After dialysis, the volume is adjusted to near the original concentration of the γ-globulin by freeze-evaporation. In addition to removing undesirable chemicals in the preparation, dialysis proves that the therapeutic agent is tightly bound to the γ-globulin since the colour of this persists after dialysis. Further proof of this tight binding is provided by precipitation of the γ-globulin with ethanol, desalting or 15 per cent trichloroacetic acid (TCA), all of which give a dark brown protein precipitate and clear supernatant. This precipitate retains the colour after extensive washing. The methotrexate-coupled γ-globulin has the expected R_F value on paper electrophoresis for γ-globulin in addition to the characteristic colour, ultra-violet absorption and fluorescence of methotrexate. These same properties were observed on precipitation bands in agar obtained when the coupled immune γ-globulins were reacted with the corresponding antigens.

Attempts are now being made to couple the same compounds on active antibody sites obtained from proteolytic and sulphitolytic treatment of the γ-globulins.

Deproteinization by TCA can be used as a method to determine quantities of unbound therapeutic compound.

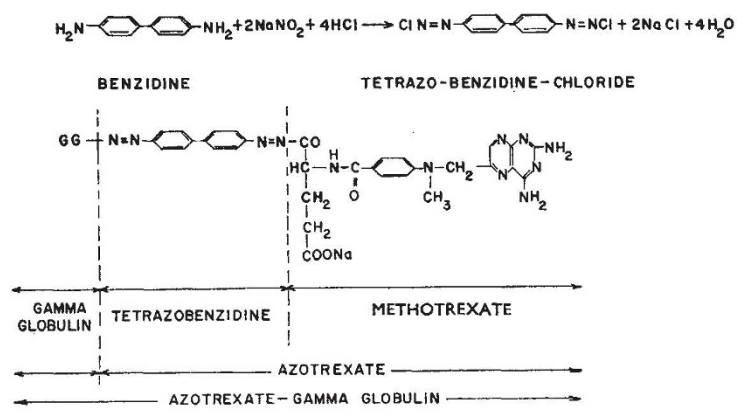


Fig. 5. Coupling of methotrexate to horse γ-globulin by diazotization (gazotrexate)

Sterility, safety, purity, pyrogenicity tests and vialing are performed by conventional methods[6].

We thank Dr. R. Duschinsky for his comments.

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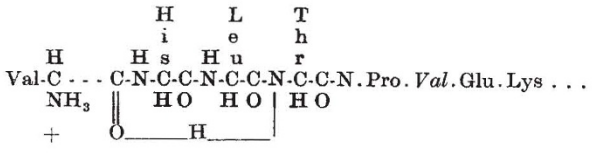
A MOLECULAR MECHANISM OF SICKLED ERYTHROCYTE FORMATION

By Dr. MAKIO MURAYAMA

Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

A SUB-MOLECULAR mechanism for gel formation in sickle-cell hæmolysate was presented in a previous report[1]; it was suggested that at 38° the amino-terminal valyl residue interlocks with the genetically interchanged valyl residue in the β-chain, allowing cyclization from carbonyl of the first valyl to the NH of the fourth threonyl by hydrogen bonding as shown schematically here:



This suggestion was made in order to account for the change in optical rotatory dispersion with temperature. The adequacy of the proposed conformation could be further evaluated by using a simple hydrophobic molecule resembling the valyl side chain, propane. I wish to report here that propane, ethane and methane were found to 'unsickle' sickled erythrocytes containing Hb-*S* (sickle-cell hæmoglobin). These compounds can also prevent and/or reverse the gel formation of deoxygenated Hb-*S* hæmolysate at 38°. The magnitude of the optical rotation is reduced by these compounds also. These observations are consistent with the hypothesis previously presented for a sequence of events which led to sickled erythrocyte formation.

Conventional methods were used to observe microscopically the phenomenon of sickling. Erythrocytes were deoxygenated using a 4 per cent sodium metabisulphite ($Na_2S_2O_5$) aqueous solution added to blood from patients known to be homozygous for Hb-*S*. A small aliquot of blood was placed in a 50-ml. round-bottomed flask which was fitted with a stopper and tubing connected to a propane tank. Propane was allowed to dissolve under a pressure of about 300 mm mercury at 38°. The gel-formation experiment at 38° was performed in a small vial fitted with a serum-bottle rubber cap pierced by 2 hypodermic needles as previously described[2]. The optical rotatory dispersion data were obtained on the instrument used in the previous report[1].

No sickling was observed when the blood specimen from a patient known to be homozygous of Hb-*S* was treated with propane. Several samples were used in this investigation, each specimen from a different patient. In the untreated aliquots serving as controls nearly 100 per cent sickling was found when deoxygenated with sodium metabisulphite. Essentially no sickling was observed when methane or ethane was substituted for propane. These simple molecules can also liquefy the gel of deoxygenated Hb-*S* hæmolysate and also prevent its formation. The changes in optical rotation at 38° normally observed in Hb-*S* hæmolysate are reduced in the presence of propane. Hb-$C_{Georgetown}$ containing erythrocytes do not 'unsickle' when treated with propane.

A sub-molecular mechanism of sickled erythrocyte formation was investigated because it was observed that a deoxygenated Hb-*S* hæmolysate of sufficient concentration gels at 38°, but liquefies reversibly on cooling to about 0° (ref. 2). Hæmolysates of normal hæmoglobin (Hb-*A*) do not gel at all. The fact that the Hb-*S* hæmolysate gel melts reversibly suggested that the molecules must undergo a dynamic conformational (architectural) change of some sort with temperature. The optical rotatory dispersion study revealed that the amplitude of the Cotton effect in the green region as well as in the Soret region is increased reversibly four-fold when the deoxygenated Hb-*S* solution is warmed to 38°. The optical rotation of a solution of Hb-*A*, on the other hand, was found to change but slightly with temperature.

It is known that the only chemical difference between the normal hæmoglobin (Hb-*A*) and the sickle-cell hæmoglobin (Hb-*S*) is in one amino-acid residue in the β-chain[3,4]. The locus of the genetic alteration is in the 6th residue from the *N*-terminus. The α-chain of both hæmoglobins has the same amino-acid sequence. The amino-acid sequence of the first 8 residues from the amino terminus of the β-chains for Hb-*A*, Hb-*S*, Hb-*C*, Hb-*G* and Hb-$C_{Georgetown}$ is as follows:

Hb-*A*	Val.His.Leu.Thr.Pro.Glu.Glu.Lys . . . .
Hb-*S*	Val.His.Leu.Thr.Pro.*Val*.Glu.Lys . . . .
Hb-*C*	Val.His.Leu.Thr.Pro.*Lys*.Glu.Lys . . . .
Hb-*G*	Val.His.Leu.Thr.Pro.Glu.*Gly*.Lys . . . .
Hb-$C_{Georgetown}$	Val.His.Leu.Thr.Pro.Glu.*Lys*.Lys . . . .

While electrophoretic mobility differences observed among hæmoglobins can be explained by the genetically interchanged amino-acid, a striking property like the phenomenon of sickling cannot be deduced so readily. It appears from the scale-model building experiments that the amino terminal valyl residue interlocks (or intercalates) with the genetically introduced valyl residue at position 6 in Hb-*S* molecule, allowing cyclization of the peptide chain from the carbonyl of the first residue to the NH of the fourth by hydrogen bonding as shown schematically in Fig. 1.

The intramolecular bond between the valyl side chains and the hydrogen bond as shown in Fig. 1 could stabilize at least 6 residues, thus restricting the freedom of rotation about bonds in the rings thus formed. This is the condition necessary for one of the Kauzmann and Eyring rules which states[5]: "Those influences which tend to restrict freedom of orientation about bonds will tend to increase the order of magnitude of the optical activity". At 0°,