CIBA FOUNDATION SYMPOSIUM

Jointly with

THE INTERNATIONAL UNION OF
BIOLOGICAL SCIENCES

ON

BIOCHEMISTRY OF HUMAN GENETICS

Editors for the Ciba Foundation

G. E. W. WOLSTENHOLME, *O.B.E.*, M.A., M.B., M.R.C.P.

and

CECILIA M. O'CONNOR, B.Sc.

With 60 Illustrations

LONDON
J. & A. CHURCHILL LTD.
104 GLOUCESTER PLACE, W.1
1959

characteristics of hereditary blocks in gal-1-P uridyl transferase we have extended our search to micro-organisms. It has been known for some time, mainly through the work of the Lederbergs, that certain K_{12} strains of *Escherichia coli* are unable to metabolize galactose and are also unable to grow on galactose as the sole carbon source—so called gal-mutants (Morse, Lederberg and Lederberg, 1956). The many gal-mutants had so far only been characterized genotypically. The challenge was to try to characterize them phenotypically and more specifically with respect to the supposedly primary gene products, the enzymes.

Kurahashi in our group first undertook to apply the enzymic techniques used in the identification of the defect in hereditary galactosaemia to bacterial mutants (Kurahashi, 1957). It soon became apparent that there were at least four genotypically different mutants which were phenotypically identical, i.e. they all had a practically complete defect in transferase. Likewise, there were three different mutants [different "mutons" in same "cistron" (Benzer, 1957)] which phenotypically could be characterized as "galactokinase-less". Finally, there are various types of single mutants (Lederberg, 1958) "leaky" with respect to the two enzymes mentioned (i.e. greatly lowered activity as compared with the wild type) and more or less completely defective with respect to the 4-epimerase (Kalckar, Kurahashi and Jordan, 1959).

Characteristic for the genotypes which are unable to synthesize gal-1-P uridyl transferase is a phenomenon which we call "galactose-induced sensitivity" because the cells react like cells in an unbalanced state only if galactose permease and galactokinase are induced (Kurahashi, 1957; Kalckar, Kurahashi and Jordan, 1959; Yarmolinsky, Kalckar and Wiesmeyer, 1959). Under these conditions, gal-1-P accumulates in large amounts just as in the afflicted children mentioned above. In most of the *Esch. coli* strains with which we have been working (Lederberg's K_{12} galactose mutants) the abnormal state could be called "galactose-induced bacteriostasis" because the cells do not lyse and they remain viable

REFERENCES

ANDERSON, E. P., KALCKAR, H. M., and ISSELBACHER, K. J. (1957). *Science*, **125**, 113.

BENZER, S. (1957). *In* Chemical Basis of Heredity, p. 70, eds. McElroy, W. D., and Glass, B. Baltimore: Johns Hopkins Press.

BERGREN, W. R., DONNELL, G., and KALCKAR, H. M. (1958), *Lancet*, **2**, 267.

BRETTAUER, R. K., HANSEN, R. G., DONNELL, G., and BERGREN, W. R. (1959). *Proc. nat. Acad. Sci. (Wash.)*, **45**, 328.

FOLCH-PI, J. (1955). *In* Biochemistry of Developing Nervous System, p. 125, ed. Waelsch, H. New York: Academic Press.

FUKASAWA, T., and NIKAIDO, H. (1959). *Nature (Lond.)*, in press.

GINSBURG, V., and NEUFELD, E. (1957). *Abstr. Proc. Biol. Chem., Amer. chem. Soc.*, September, 27 C.

HOLZEL, A. (1959). *In* The Geigy Symp., Bern, 1958.

HOLZEL, A., and KOMROWER, G. M. (1955). *Arch. Dis. Childh.*, **30**, 155.

ISSELBACHER, K. J. (1957). *Science*, **126**, 652.

ISSELBACHER, K. J., ANDERSON, E. P., KURAHASHI, K., and KALCKAR, H. M. (1956). *Science*, **123**, 635.

KALCKAR, H. M. (1959). *Nature (Lond.)*, **183**, 1266.

KALCKAR, H. M., ANDERSON, E. P., and ISSELBACHER, K. J. (1956*a*). *Proc. nat. Acad. Sci. (Wash.)*, **42**, 49.

KALCKAR, H. M., ANDERSON, E. P., and ISSELBACHER, K. J. (1956*b*). *Biochim. biophys. Acta*, **20**, 262.

KALCKAR, H. M., BRAGANCA, B., and MUNCH-PETERSEN, A. (1953). *Nature (Lond.)*, **172**, 1039.

KALCKAR, H. M., KURAHASHI, K., and JORDAN, E. (1959). *Proc. nat. Acad. Sci. (Wash.)*, in press.

KIRKMAN, H. N. (1959*a*). *Ann. hum. Genet.*, **23**, 117.

KIRKMAN, H. N. (1959*b*). *Nature (Lond.)*, in press.

KIRKMAN, H. N., and KALCKAR, H. M. (1958). *Ann. N.Y. Acad. Sci.*, **75**, 274.

KURAHASHI, K. (1957). *Science*, **125**, 114.

LEDERBERG, E. (1958). *X Int. Conf. Genet., Montreal.*

LELOIR, L. F. (1951*a*). *In* Phosphorus Metabolism, **1**, 762, eds. MCELROY, W. D., and GLASS, B. Baltimore: Johns Hopkins Press.

LELOIR, L. F. (1951*b*). *Arch. Biochem.*, **33**, 186.

LELOIR, L. F. (1955). *Proc. III Int. Congr. Biochem.*, p. 154.

LERMAN, S. (1959). *A.M.A. Arch. Ophthal.*, **61**, 88.

MAXWELL, E. S. (1957). *J. biol. Chem.*, **229**, 139.

MAXWELL, E. S., KALCKAR, H. M., and BURTON, R. M. (1955). *Biochim. biophys. Acta*, **18**, 444.

MORSE, M. L., LEDERBERG, E. M., and LEDERBERG, J. (1956). *Genetics*, **41**, 143, 758.

MUNCH-PETERSEN, A., KALCKAR, H. M., and SMITH, E. E. B. (1955). *Biol. Medd. (Kbh.)*, **22**, 3.

NAJJAR, V. (1959). *In* The Geigy Symp., Bern, 1958.

DISCUSSION

Lederberg: Dr. Kalmus, would you comment on the Land phenomenon?

Kalmus: A fully coloured picture is produced by two colour components only when it is of some complexity; it depends on the interaction of retinal neighbouring areas and possibly on central integration; but also on the faculty of all cone types of responding to a wide range of wavelengths if the light is of sufficient intensity. The existence of separate cone mechanisms remained speculative for over 100 years until Rushton's demonstration. Subjectively two of the mechanisms can be shown quite easily. Any normal person can for instance be made a temporary protanope in half a minute by exposing his eyes to very strong red light. He will then be unable for a few seconds to read certain Ishihara charts. If he bleaches only one of his eyes with red light his binocular colour match on the anomaloscope will contain a huge excess of red to match the standard yellow. Temporary deuteranopia can similarly be produced by green bleaching. Even more convincing is a demonstration of the separate various cone mechanisms in Stiles' adaptation experiments. If an intermittent blue light signal is matched against a permanently orange illuminated background the intensity of which can be increased, the colour of the flashing light at the threshold changes and a kink occurs in the curve.

Smithies: Dr. Kalmus, did you say there are some people colour blind in one eye who have normal vision in the other?

Kalmus: About sixty such people have so far been described. One such woman has helped to produce a film which I showed in 1958 at the Genetical Congress in Montreal, demonstrating what the world looks like to a colour blind person. By matching ordinary items of an interior scene, as seen with her defective eye, to similar items as seen with her normal eye she found that she had to remove all greens and reds so that only blue and yellow of various brightness and saturation remained in her visual world.

Smithies: Was this an acquired colour blindness in one eye?

Kalmus: It is the ordinary type of deuteranopia; but others as well as myself have tried in vain to get information about her family. In general, family histories of people colour blind in one eye found in the literature are badly reported but in some the condition seems to have occurred in both eyes in some relationship.

Kalckar: It would be interesting to see whether the Land phenomenon could be elicited in colour blind people.

Kalmus: I have produced the phenomenon in a deuteranopic colleague; but in my paper I purposely dealt with receptors especially

have mentioned, such substances occur in many higher plants such as cabbages and turnips.

Lederberg: There is a statement in several textbooks to the effect that the differential response to phenylthiourea depends on its being dissolved in saliva rather than water. Is that true?

Kalmus: Several years ago we did some rather disgusting experiments on this point, but I could never find anything positive. I think it is not true, but I am not sure.

year. This has been done by Alving and his group, and in a fairly short time the haematocrit rises to normal levels, so that it is unlikely that the administration of antimalarials is a very great evil as a selective agent.

Ingram: Does quinine give this effect?

Childs: Apparently very little.

Lederberg: Have you any more to say about the mechanism of haemolysis? You said that oxidants had something to do with it.

Childs: The reasoning that led to the discovery of abnormality of glutathione and G-6-PD was that the injurious agents might, after they got into the cell, become oxidized and act as oxidizing agents. In an attempt to find something which might protect against oxidation, glutathione was tried. But very little is known about the mechanism of haemolysis.

Smithies: Dr. Childs, you say that prolonged administration of the antimalarials does not appear to produce any lasting serious effect and so makes it difficult to see what selective forces are involved in changing the gene frequencies, but the population in which these tests were made is well nourished. In a population where there is, for example, a possibility of iron deficiency there might be some selection against individuals in whom haemolysis was constantly occurring.

Penrose: The real problem here is that practically all the individuals with this enzyme deficiency are at a disadvantage. They get favism with the bean and they get haemolysis if they take an antimalarial drug. How is it possible for such a gene to be so prevalent? I think that is the problem which many of us have in mind and which Dr. Motulsky examined very critically. If the gene gives protection against malaria, a high incidence in Sardinia would be suggestive but, as Dr. Childs says, it would be very important to make more surveys in other districts which have been malarious. I don't know what the details are of other European or Mediterranean countries where there is a lot of malaria, but I understand that in the eastern Mediterranean there are quite a number of communities which have a high incidence of this enzyme deficiency, and this rather suggests that there might be a real advantage there. If it is true that favism is less severe in the female than in the male, the female heterozygotes have some advantage.

Ceppellini: I have already spoken of the so-called Mediterranean disorder, which implies thalassaemia, favism and also a haemolytic disease which is probably congenital, non-spherocytic anaemia. These have the same distribution pattern. As regards an advantage, if we accept malaria—and I don't say that we must accept malaria—it is reasonable to think that any disturbance of red cells will be

advantageous, because probably there is normally a perfect symbiosis between the parasite and the host; therefore in thalassaemia and similar disorders the defects can all have the same effect because they disturb the symbiosis.

Lederberg: Dr. Childs, could you comment on Marks' report that the juvenile erythrocytes are normal and the older ones are not?

Childs: Marks has shown that in the young cells from a sensitive person the enzyme activity is high. In reticulocytes, for example, the enzyme assay is pretty close to normal. So the defect is one which is found to a greater degree in the older cells and might in a sense be regarded as premature ageing.

Lederberg: Then does an age-sensitive "mutant enzyme" replace a normal component, or are both present in normal cells, the mutant cells having only the age-sensitive component? How do *in vitro* preparations of the enzyme behave with respect to loss of activity on ageing? One should study preparations from young cells whose activity would not already have been lost.

Childs: This has not been studied so far as I know. I think it would be worth investigating it in individuals after a haemolytic episode where there was a large amount of reticulocytes, or in patients who have haemolytic anaemia and who are always in a phase of reticulocytosis.

Ceppellini: A problem which is certainly not new in this case, but which can be raised for any sex-linked defect is why is the hemizygous normal male better off than the heterozygous female? If we assume that the mutant gene is a complete amorph then there should be some kind of dosage effect. In fact, the heterozygous female has less activity than the male. I think this is the real problem of alleles.

Childs: This has not been done in the formal sense. Until there are more data and a more precise method, it is very hard to comment.

Cavalli-Sforza: Dr. Siniscalco, on the question of distribution of gene frequency in connexion with malaria: in the eastern area of Sardinia where you said that the frequency of primaquine sensitivity is small, I understand there is a very high incidence of thalassaemia and I wonder whether the incidence of primaquine sensitivity might be negatively correlated with the frequency of thalassaemia, in those areas of Sardinia where malaria has been prevalent.

Siniscalco: So far, there are no reliable population data on thalassaemia for the west side of Sardinia. This is a very important problem, and we are going to study the distribution of thalassaemia and also the distribution of the enzyme deficiency in thalassaemic populations already studied, such as in the district of Ferrara.

Ceppellini: Marcolongo speaks of D.E.M. (haemotological Mediterranean disorders) which include the thalassaemias, favism, some

SINGER, S. J., and ITANO, H. A. (1959). *Proc. nat. Acad. Sci. (Wash.)*, **45**, 174.

SMITH, E. W., and TORBERT, J. V. (1958). *Bull. Johns Hopk. Hosp.*, **101**, 38.

THORUP, O. A., ITANO, H. A., WHEBY, M., and LEAVELL, B. S. (1956). *Science*, **123**, 889.

VINOGRAD, J., HUTCHINSON, W. D., and SCHROEDER, W. A. (1959). *J. Amer. chem. Soc.*, **81**, 3168.

DISCUSSION

Burnet: Have you any information about the serological differences amongst the various haemoglobins?

Itano: Foetal haemoglobin is easily distinguished serologically from adult haemoglobin; however, haemoglobins A and S are difficult to distinguish. Goodman and Campbell using both rabbit and chicken antisera have reported evidence for minor differences in antigenicity between haemoglobins A and S [Goodman, M., and Campbell, D. H. (1953). *Blood*, 8, 422].

Lederberg: Have you looked for iso-antibodies after transfusion in Man?

Itano: No.

Lederberg: Would that not be the most sensitive method of looking for a difference between the haemoglobins?

Ceppellini: But there is absolutely no evidence of iso-antibodies in Man; they are only in rabbits.

Lederberg: That is what you would expect. The two proteins are almost identical and quite different from the rabbit's.

Ceppellini: But S and A are too similar to be antigenic. I agree that isoimmunization is always more sensitive.

Rossi-Fanelli: I wonder if, following acid dissociation, studies have been made of the physiological properties of the reconstituted haemoglobin, for example, the combination with oxygen or other substrates, because acid dissociation may alter the molecule. Secondly, have you tried dissociating with urea?

Itano: Treatment with acid at low pH does alter the molecule, and the alteration is accompanied by a marked change in absorption spectrum to that of acid-denatured haemoglobin. Dissociation into two subunits at pH 4–6 appears to be reversible according to physical criteria, and the reconstituted molecule has the electrophoretic mobility and absorption spectrum of native haemoglobin. We have not examined the physiological properties of reversibly dissociated haemoglobin, nor have we attempted recombination experiments with urea-dissociated haemoglobin. It has been my experience in the past that removal of urea from a solution of urea-dissociated

haemoglobin A results in precipitation of a large fraction of the haemoglobin; therefore dissociation with urea is probably not as readily reversible as dissociation with dilute acid.

Brenner: Have you done any recombination experiments with haemoglobin M?

Itano: I have not worked with haemoglobin M although the properties of one of the two chemically different forms of haemoglobin M suggest the application of recombination experiments. About half of the haem groups in this haemoglobin have abnormal chemical reactivities [Gerald, P. S., and George, P. (1959). *Science*, **129**, 393]. It would be possible to determine whether these abnormal haem positions are associated with a particular pair of chains by recombining haemoglobin M with abnormal haemoglobins in which the defective chain has been identified.

Lederberg: Dr. Itano, you referred to the Hopkins-2 family as evidence of interchange of chains *in vivo*. Could you clarify the argument?

Itano: The familial evidence published by Smith and Torbert shows that haemoglobins S and Hopkins-2 are controlled by genes that segregate independently. According to their electrophoretic analyses individuals with both abnormal genes have three haemoglobin components, the two abnormal forms and haemoglobin A. On the other hand, individuals with haemoglobins S and C, which are chemically defective in the same chain and which according to familial studies are under the control of allelic genes, do not have any haemoglobin A. Our recombination experiments suggest that Hopkins-2 is abnormal in the α chain. The findings of Smith and Torbert can be reconciled with the structural composition of the haemoglobin molecule if we postulate that the component that migrates like haemoglobin A in apparent double heterozygotes includes, in addition to haemoglobin A, a double abnormal molecule composed of the abnormal β chain of haemoglobin S and the abnormal α chain of haemoglobin Hopkins-2, the charge defects of the abnormal chains cancelling out to produce a new species with the same net charge as haemoglobin A. Since we are looking at haemoglobin after completion of synthesis, we cannot state whether the apparent formation of four molecular species occurs by synthesis of two different pairs of α chains and two different pairs of β chains followed by random association of these pairs or by synthesis of haemoglobin S and Hopkins-2 followed by intracellular interchange of chains. The familial evidence shows that the genes for S and Hopkins-2 segregate independently; therefore it would appear more likely that synthesis of the different chains on independent templates precedes random association.

Lederberg: The very fact that there are more than two components is the basis of your argument?

Itano: The presence of more than two components and the mobility of the third component, which suggests that it may be a mixture of haemoglobin A and a doubly abnormal molecule, are the chemical bases of my argument. It will be necessary to isolate the third component and examine it chemically for the presence of both abnormal chains in order to test my hypothesis.

to be very low: in fact his is intermediate, more like a mixture of A and S, or S with some other haemoglobin, like G. It is conceivable that in this case a second component is present due to an excess of α chains being made. This person would then have both haemoglobin S and a new and unknown component—α_4^A, which electrophoretically would be almost indistinguishable from haemoglobin S, but would have quite a different solubility and explain the fact that the solubility of haemoglobin in this case is not low as it ought to be. When one looks at the published pictures of paper and boundary electrophoresis, this hypothesis is not unreasonable; there are certain bumps and shoulders in the diagrams. However, the brother of the propositus has *no* thalassaemia, his genotype would be β^S/β^G and again the electrophoretic and solubility data, etc., are consistent with the view that he does indeed have both haemoglobin G and S, and no α_4^A, no thalassaemia. The reason that the various haemoglobins in this family have been so difficult to distinguish chemically is due to the unfortunate circumstance that both haemoglobins G and S and the as yet hypothetical α_4^A have very similar electrophoretic mobilities.

Kalmus: Dr. Ingram might perhaps find time to look at some of the mouse literature. I think anaemias have been described in laboratory mice affecting the foetal, intermediate and adult generations of red cells both morphologically and in respect of their haemoglobin content. I should also like to know whether Dr. Ingram has any particular reason for using only trypsin to study variation in the α chains, and not other enzymes?

Ingram: There are other enzymes which can be used in this work, but they are not as specific as trypsin, and that means that you are likely to get apparent variations due to the presence of the products of incomplete digestion. Trypsin is still the best.

Lederberg: With regard to the chemical methods of determining allelism, in principle the unique products of mutant genotypes might have been specified already in the wild type. Does the mutation convey new information or switch the actual production to another locus already there? This seems to hold for the alternative production of haemoglobins A and F. One relevant datum would be the possible occurrence of trace amounts of mutant haemoglobins in "normal" bloods. I do not mean at all that this argument should be taken very seriously but it has to be considered in a rigorous analysis. What is the maximum level of alternative haemoglobins that could be present in the normal individual and yet not be detected by existing methods—is it as high as 1 per cent?

Ingram: I would guess that it is of the order of between one-tenth and one per cent.

Lederberg: Do you think it would be worth while to make a systematic search for residual amounts of variant haemoglobin?

Ingram: This is being done at the California Institute of Technology. There are a number of minor components of haemoglobin A apart from the two I have already mentioned which do occur and which are being investigated. There are, however, other ways in which these minor components could arise. The whole series of minor components is apparently repeated in haemoglobin S and haemoglobin C; in other words, it is possible that those are errors in the mechanism of production.

Lederberg: In the α chain, for example?

Ingram: Yes, they could be systematic errors. Or they could be different assortments of α and β chains. We have only heard so far of haemoglobins which are $\alpha_2^A\beta_2^A$ and β_4^A and α_4^A, but this is because nobody has looked for $\alpha_1^A\beta_3^A$ and $\alpha_3^A\beta_1^A$, etc.

Lederberg: Has β_4 been obtained by reassociation *in vitro*? Is β_4 an hypothetical or a real substance?

Ingram: β_4^A is a real substance, it is haemoglobin H. Schroeder and Vinograd tell me that they have made β_4^S.

Brenner: No one has separated the half-molecules?

Ingram: Not yet.

Lederberg: You also have somatic mutation, of course, in the erythropoietic system, which might be expected.

Ingram: You would expect to find those in the series of minor components which change with the adult.

Lederberg: Then we can justify the idea that there are many genes controlling the series of haemoglobins. Now what criterion do you use to relate S to A as being the mutant of that particular β chain?

Ingram: This is purely through genetical studies.

Lederberg: You use genetical evidence after all to determine allelism!

Pontecorvo: There are certain replacements of amino acids which will give an electrophoretic difference and others which will not. What proportion of possible changes, if every amino acid could be changed, would be electrophoretically detected?

Brenner: If you were given a protein which could change at random, and if all amino acids occurred equally frequently in that protein, then 40 per cent of all possible changes would lead to charge differences. This would be subject to a scaling factor depending on the composition.

Ingram: Does this include changes in uncharged amino acids?

Brenner: Yes, all possible changes.

Smithies: It is of interest that in cattle three transferrins can be

Ingram: I would be happy with any number of thalassaemia loci up to two!

Harris: With regard to the hypothesis that Dr. Ingram has put forward, the point as I understand it is that if you have the sickle-cell gene and this kind of thalassaemia gene together, the chances are you will get an extra component. I also understand that many examples of sickle-cell thalassaemia disease have been reported. Is there any indication of such a component in all the quite large number of cases which have been described?

Ingram: No, but that has not been looked for.

Lederberg: What about the father of the persons indicated?

Ingram: The father was SA.

Lederberg: This could be another type of recombination product. If this is a *T* gene which blocks the formation of the α chain it may still be able to form a recognizable haemoglobin in combination with the β chain of haemoglobin S. That, I think, is what you mean by a "hybrid substance".

Ingram: This is also a possibility, but I am not sure whether you could do this background for the whole family.

Lederberg: What exactly was the evidence that H is β_4^A?

Ingram: The evidence is partly the type of hybridization experiment which Dr. Itano talked about, compatibility with β_2^A. Also by chemical analysis which shows that all the peptide chains of haemoglobin H begin with val–his–leu which is the beginning of the β chain, and not with val–leu which is the α chain.

Lederberg: When haemoglobin H is dissociated there is no component which will then combine with the β chain?

Ingram: That is correct.

Lederberg: But when it is dissociated it also reassociates?

Ingram: Yes; and also if you take haemoglobin H and haemoglobin S and recombine you get a new haemoglobin which moves close to the normal.

Montalenti: Some electrophoretic patterns of haemoglobins have been obtained in my laboratory by Drs. Adinolfi, Chieffi and Siniscalco, in the lamprey, one of the lowest vertebrates [Adinolfi, M., and Chieffi, G. (1958). *Nature* (*Lond.*), **182**, 730; Adinolofi, M., Chieffi, G., and Siniscalco, M. (1959). *Nature* (*Lond.*), in press]. Larval haemoglobins have been discovered also in fishes, both in Teleosts and in Elasmobranchs. Fig. 1 illustrates the difference in the haemoglobin patterns of the brook lamprey *Petromyzon planeri* during the course of development. The diagram shows the four haemoglobin components in this lamprey, two of which are typical of the larval stage and two of the adult stage. Ammocoetes of *P. planeri* can be found throughout the year in the River Sarno near Naples and they always

patterns in the lamprey are of some physiological importance and possibly connected with the morphological changes which take place during metamorphosis and sexual maturation.

Siniscalco: It is worth mentioning, to show that we are not dealing with an electrophoretic artifact, that each of the haemoglobin components when isolated and subjected to a second starch gel electrophoresis preserved unaltered its identity and electrophoretic properties. The multiple pattern of lamprey haemoglobin is also obtainable on starch block and on paper electrophoresis (especially acetate-cellulose paper). It seems therefore quite likely that the separation in different haemoglobin bands is due to differences in the net charge more than to differences in molecular size.

If this is so, and bearing in mind that lamprey haemoglobin seems to consist only of molecules with a single haem, we feel that fine structure analysis might very profitably be used to elucidate more closely this rather complicated situation.

Finally it must be added that there was no obvious genetical polymorphism either in the 119 ammocoetes or the 102 adults so far investigated from the River Sarno, and that exactly the same adult haemoglobin pattern was observed in adult individuals of *P. fluviatilis*, a species closely related to *P. planeri*, which lives in the sea during the larval stage and then comes up to the fresh water to reach maturity.

Brenner: What is the evidence for one haem?

Siniscalco: The molecular weight in the sea lamprey has been measured and is about 16,000 [Wald, G., and Riggs, A. (1951). *J. gen. Physiol.*, **35**, 45; Lenhert, P. G., Lowe, W. E., and Carlson, F. D. (1956). *Biol. Bull. (Wood's Hole)*, **111**, 293].

Brenner: Have you measured the oxygen equilibrium? Do you get haem-haem interaction?

Siniscalco: No. As I said, we have to go further with the physiochemical investigations. But no haem-haem interaction was found by Wald and Riggs (1951, *loc. cit.*) in the sea lamprey *P. marinus*.

Brenner: This may be very important from the evolutionary point of view, and it may well be important to investigate the two separate components.

Lederberg: Is there some adaptive significance in the shift of haemoglobins?

Siniscalco: This is what we wonder. Evidently there must be something which is connected with the switch from the synthesis of one type to that of the other. All we can say is that when the adult type of haemoglobin appears two other important phenomena occur: individuals undergo metamorphosis, ultimately reaching sexual maturity, and they leave the river bed, where they have been through the whole larval stage, to swim freely in the water.

one did not have to postulate a separate locus for a suppressor effect. However, with respect to the ahaptoglobinaemia situation, Dr Sutton and I have recently studied a family in which, because of the haptoglobin types in the family, it can be shown rather clearly that ahaptoglobinaemia (which is not complete) was due to a locus separate from the haptoglobin locus.

Lederberg: Is anything known of the clearance of haptoglobin transfused into individuals showing a defect in its production?

Harris: We do not know about it in the haptoglobins, i.e. we do not know whether ahaptoglobinaemia is due to the rate of synthesis or rate of destruction, but in analbuminaemia and afibrinogenaemia it is quite clear. The odd thing is that in some of the analbuminaemics and agammaglobinaemics the rate of destruction of the normal gamma globulin or normal albumin may be slower than in normal individuals.

Burnet: As regards this question of traces of normal proteins that are always present, is it possible that these represent back mutations in limited proportions of cells?

Harris: This is a further hypothesis and obviously a plausible one. I mentioned three or four others, but I do not think there is much evidence one way or the other for any of them.

Siniscalco: Concerning the occurrence of cases of ahaptoglobinaemia, it might be worth while repeating, at a different time of the year, the typing of individuals found to lack haptoglobins, in order to exclude the possible influence of seasonal factors on the rate of destruction of those plasma components.

As I pointed out during the discussion of Dr. Child's paper we found that, in Sardinia, several cases of sera showing a weak haptoglobin pattern were detectable during the spring and we have reason to believe that this phenomenon is due to *in vivo* haemolysis occurring in individuals with 6-G-PD deficiency who are exposed in one way or another to the toxic effect of fresh fava beans. With this in mind I wonder if those population data from Africa, where an incidence of 20–30 per cent of zero-types has been found, were all collected during the summer and, if not, whether the incidence of these zero-types was constant throughout the year. Of course, information on the sex distribution among those individuals and family data would also be very important for testing the hypothesis that 6-G-PD deficiency, together with some environmental toxic factors, might be responsible for the disappearance of haptoglobins from the blood stream. In this connexion it may be remembered that 30 per cent of the male population in some areas of the Belgian Congo was found by Dr. Motulsky (personal communication) to show this defect.

Ingram: The whole subject of electrophoretic changes which can be ascribed to amino acid constitution is unclear at the moment, but all possibilities should be considered. If you change the charged amino acids you get the corresponding electrophoretic change for the whole protein. You can also postulate that if you change certain uncharged amino acids only the structure will be altered in such a way that also you get electrophoretic differences due to covering or uncovering charged groups or altering their pK's through changes in their environment. As regards haemoglobin E, it is safe to say that we cannot completely correlate the observed amino acid charge difference with the behaviour of the intact protein. I personally think there are two effects, a primary effect of amino acid substitution and secondary effect in which this substitution has altered the architecture of the protein.

Smithies: Yes, I agree with this completely. It is just a generalization that if one regards the electrophoretic differences as due primarily to electric charge, then one has to assume that at least 4 amino acid residues must be variable in the transferrins. However, if there are secondary effects then this will reduce the number of amino acids which must be presumed to vary.

Lederberg: Dr. Ingram, do you think it worth while to induce further electrophoretic differences, for example by removing free amino groups with nitrous acid?

Ingram: No, I don't think so, and one of the reasons is that such chemical alterations are not quantitative, not clean enough, although in theory it is a good idea.

Brenner: We have gone into this in an attempt to find a more efficient screening method for proteins, and it seems that it can work only when there is a change in a cysteine; in this case one can really put on a charged group. One might possibly do things with serine or threonine, but the reactions are not clean. Of course, a vast number of so far undetectable changes in proteins, and which we feel could not be detected even on finger-printing, are those involving the non-polar amino acids. This presents one of the big problems of screening the molecular structure of proteins. There are only rather tedious ways around this difficulty, and it seems that one may be compelled in many cases to go even further than finger-print screening and actually determine amino acid compositions for all the peptides. This would be too much to do routinely, but automatic methods are being developed and it may soon be technically feasible.

Lederberg: Dr. Smithies, do the different genetic forms of haptoglobins have the same isoelectric point?

Smithies: Yes, I believe that they do although I know of no exact measurements to prove this. The purification procedure we have

Cavalli-Sforza: Dr. Smithies, is there information available on which substances other than haemoglobin have an affinity for haptoglobin? Is it the globin part of the haemoglobin that has an affinity for haptoglobin?

Smithies: I'm not sure that anybody has prepared globin from haemoglobin and then established that it combines with haptoglobin, but it is my impression that it is the globin part of the haemoglobin which is combining. As for other materials which have an affinity, it has been suggested that vitamin B_{12} is bound by the haptoglobins.

Ingram: Are the haptoglobins able to distinguish between the series of abnormal haemoglobins and also between haemoglobins of different species?

Smithies: I don't know of any test of this although in Detroit M. D. Poulik at one time planned to try the abnormal haemoglobins. Human haemoglobin will combine with rabbit haptoglobin, and rabbit haemoglobin with human haptoglobin, so I think that any such distinction between normal and abnormal haemoglobins by the haptoglobins is unlikely.

Lederberg: Is it known that haptoglobin does *not* contain the same α or β chains as haemoglobin?

Smithies: I don't think it is known. This might be rather interesting, because perhaps haptoglobin can combine with haemoglobin since it does in fact contain the same amino acid sequence as the α or β chain.

Brenner: You then have to postulate that in the presence of haptoglobin the haemoglobin dissociates.

Smithies: I think it will dissociate, because (although we believe that haptoglobin type 1–1 normally combines with one molecule of haemoglobin) when insufficient haemoglobin is added (i.e. less than will saturate the haptoglobin) you get an intermediate complex which migrates in starch gels more slowly than uncombined haptoglobin but faster than fully saturated haptoglobin (Laurell, C. B. (1959). *Clin. chim. Acta*, 4, 79).

This suggests that haptoglobin can pick up a half-molecule of haemoglobin (by dissociating haemoglobin) when there is insufficient haemoglobin to saturate all the haptoglobin.

Ingram: It is known that globin, which is a half-molecule, does polymerize; it forms polymers and precipitates perhaps a series of them as in the haptoglobins. But one would then expect that haptoglobin should combine with haem.

Smithies: Is it known whether the part of haemoglobin that causes the α and β chains of the haemoglobin to combine together is the same part that combines with haem?

Ingram: That is not known.

Smithies: Then it would not necessarily follow that haptoglobin would have to combine with haem.

Itano: It is doubtful that haem is the site at which haptoglobin is bound to haemoglobin. Large ligands are bound to haem with difficulty and tend to denature haemoglobin after they are bound. The suggestion that haptoglobin is the same as globin can be tested by adding less than an equivalent amount of haem to globin and looking for a molecule that has the properties of a haemoglobin-haptoglobin complex. As for the proposal that one of the complexes is composed of haptoglobin and a half-molecule of haemoglobin, the presence of only one such complex suggests either that this particular half-molecule is a product of symmetrical dissociation or that only one of the two products of asymmetric dissociation is capable of associating with haptoglobin.

Lederberg: What is known of the haptoglobin in the individuals who are homozygous for abnormal haemoglobin; have they been looked at?

Smithies: I don't know what they are like or, indeed, if they have been examined.

SMITHIES, O. (1955). *Biochem. J.*, **61**, 629.
SMITHIES, O., and WALKER, N. F. (1956). *Nature (Lond.)*, **178**, 694.
WATKINS, W. M. (1956). *Biochem. J.*, **64**, 21P.
WATKINS, W. M., and MORGAN, W. T. J. (1955*a*). *Vox Sang. (Basel)*, **5**, 1.
WATKINS, W. M., and MORGAN, W. T. J. (1955*b*). *Nature (Lond.)*, **175**, 676.
WATKINS, W. M., and MORGAN, W. T. J. (1956). *Nature (Lond.)*, **178**, 1289.
WATKINS, W. M., and MORGAN, W. T. J. (1956–57). *Acta genet. (Basel)*, **6**, 521.
WATKINS, W. M., and MORGAN, W. T. J. (1957). *Nature (Lond.)*, **180**, 1038.
WATKINS, W. M., and MORGAN, W. T. J. (1959). *Vox Sang. (Basel)*, **4**, 97.
WHELAN, W. J. (1956–57). *Lect. Scientific Basis of Medicine*, **6**, 233.
WIENER, A. S., and WEXLER, I. B. (1952). *Bact. Rev.*, **16**, 69.

DISCUSSION

Grubb: I wonder if the gene *l′* is an amorph or if it is responsible for the appearance of a precursor substance?

Watkins: The gene *l′* cannot be responsible for the appearance of the precursor substance because, according to the scheme, individuals who do not possess this gene, i.e. individuals who are homozygous for the allele *L′*, nevertheless have precursor substance which is converted to Le^a substance by the action of the *L′* gene. It is therefore suggested that the precursor mucopolysaccharide material is formed at an earlier stage in the biosynthetic pathway and that the *l′* gene is an amorph which brings about no further change in this substance.

Lederberg: How well-ordered is the polysaccharide? Could it be understood as a product of a mélange of enzymes putting on the various side chains wherever the configurational specificities admit, or do you have to postulate a definite serial assembly?

Morgan: It is not possible to answer your question completely, because we do not yet know the detailed structure of the specific substances. We know that all the group mucopolysaccharides, irrespective of serological specificity, contain the same four sugars in roughly the same amounts, so that there is no selection from this point of view. Each substance is completely distinct serologically, and therefore there is some control that always goes on in a group A person, for example, which determines that certain of the carbohydrate chains shall end with a non-reducing α-*N*-acetylgalactosaminoyl unit attached most probably by a 1 : 3 linkage to galactose.

Watkins: We would also believe that there is a precisely determined sequence responsible for specificity.

Lederberg: Let me put it a little differently. Is there a more definite structure than could be inferred from knowing all the component disaccharide segments?

Watkins: Yes.

Lederberg: Could you enlarge on this?

Morgan: The general body of evidence concerning the immunological specificity of carbohydrate structures indicates that whereas the non-reducing end unit plays a dominant part in the serologically specific character of the material, the second and subsequent sugar units and their linkages also contribute to the specificity; the contribution of each sugar residue having a smaller and smaller influence the further they are from the serologically dominant non-reducing end unit. Of the six N-containing disaccharides isolated from group A substance, only one has any A specificity.

Watkins: And that has only a very small fraction of the total activity of the whole specific unit.

Lederberg: I am not questioning the specificity of the antibody which may well be specific for larger sequences. Does the antigen contain only such ordered segments, or are these occasional features in a more random conjunction?

Morgan: As we do not know how long the carbohydrate chains are or whether there is more than one type of chain in each specific substance, it is not possible to say if specifically ordered oligosaccharide sequences exist throughout the substance.

Lederberg: Do you know whether the blood group substance consists only of such repeating units as long, say, as four residues?

Morgan: It is not possible to say if there is a repeating tetrasaccharide unit.

Kalmus: The word precursor denotes a definite sequence of reactions in a biochemical scheme. There seems to be some doubt about the special arrangements of some of those sequences. Could not different sequences explain why different antigens occur in the same person at the surface of the red cells and in the secretions? Also could not similar syntheses be effected by simultaneous reactions?

Morgan: The ordered sequence of gene action suggested in the scheme has been put forward to account for the biochemical and serological facts. It has been found possible, for example, to degrade A and B substances enzymically and to obtain newly developed H activity whereas it has not been possible to detect A or B activity on degradation of H substance. It is therefore suggested that H is the substrate on which the *A* and *B* genes act. The order of the other steps has been suggested for similar reasons. It does not seem probable that a different order of gene action could explain why different

H activity, a large number of saliva specimens from group AB secretor persons and found occasionally a considerable variation in the amount of specific activity in individual saliva specimens. For example, a high A titre did not always go with a high H titre.

Lederberg: Dr. Watkins, are you able to degrade AB substance enzymically and leave A or B activity?

Watkins: Yes.

Lederberg: Does this involve removal of a side-chain?

Watkins: We have only been able to try this with a B enzyme because we have not obtained a sufficiently purified preparation of A enzyme. With the B enzyme, however, there is a removal mainly of free galactose with traces of fucose and *N*-acetylhexosamine as well. There is no evidence for the release of oligosaccharide units, and we believe that the enzymes which destroy the serological activity of the blood group substances function by removing the terminal non-reducing unit of the sugar sequence which determines specificity. After the action of the B enzyme and AB substance one is left with a molecule which has lost its B activity, but which retains its A activity and shows newly developed H serological properties.

Grubb: I cannot be sure of that. There is unpublished evidence that there are some different types in Negro populations.

Burnet: You mentioned other types of rheumatoid arthritis sera anti-A with entirely different specificities. Is your Gm(b) the same as Gm(a)?

Grubb: Yes.

Burnet: And that is produced with the same type of indicator Rh incomplete serum.

Grubb: You must use anti-Rh produced in Gm(a−) individuals.

Burnet: So that this a and b do represent more or less homologous types of antibody?

Grubb: Yes.

Lederberg: The sera from your rheumatoid arthritis donors show both the macroglobulin antibody and a globulin with which it reacts?

Grubb: You might find this and it appears as a prozone phenomenon.

Wright: From one of your tables I got the impression that the frequency of Gm(a+) was higher in those countries where rheumatoid arthritis may be infrequent, e.g. Africa. Is there any correlation between the distribution of these Gm types and the frequency of rheumatoid arthritis?

Grubb: There is evidence that in the area of the Equator rheumatoid arthritis is very infrequent, and you can also find statements that it is infrequent in Eskimos; I don't know more than that and I don't know what it means.

Brenner: Am I correct in saying that amongst rheumatoid arthritis cases the distribution of Gm(a+) and Gm(a−) is very similar to that in the general population?

Grubb: Yes.

PANEL DISCUSSION

Montalenti: Prof. Penrose said earlier that tissue culture is the new star in the sky of human genetics, and whether it is a star of prime magnitude or just a starlet we are now about to hear. The approach to genetical problems by tissue culture is only at the very beginning, and so we have arranged a discussion by a panel composed of Prof. Luria, Prof. Pontecorvo, Prof. Cavalli-Sforza, Prof. Eagle and Prof. Lederberg. I have asked Prof. Luria to be the moderator.

Luria: It is good that we have Dr. Eagle here, otherwise this would almost be a panel of inexperts since some of us have not done anything with tissue cultures. However, Dr. Eagle is a real expert and we shall fall back on him for factual information.

in 1936, and the amount of information we have now added makes Stern's interpretation certain. Somatic crossing over permits the identification of the location of the centromeres and of the sequence of loci in one arm of any one chromosome. As I said, we have no clue as yet as to whether or not somatic crossing over occurs in vertebrates and in particular in Man.

A second process of somatic segregation is haploidization. Consider a cell heterozygous for a number of markers on a number of chromosomes. Such a cell, by an accident of mitosis, gives origin to a cell which has lost one of its chromosomes. This puts a premium on further losses, so that in the clonal line derived from that cell, eventually either one or the other member of each of the other pairs of chromosomes is lost until finally a completely haploid set is attained. This haploid set may of course be made up of any association between the members of the various chromosome pairs. There is, therefore, recombination between whole chromosomes, and different recombinant sublines arise in the clone.

Brenner: Why do you say there is a premium on further losses?

Pontecorvo: This is an experimental fact in *Aspergillus nidulans* (which has eight chromosomes) and I believe it to be likely in other cases. If you isolate segregant cells only a few nuclear generations after segregation for one marker has occurred, you can follow the occurrence of further losses until the complete haploid set is attained. On the other hand, another compensatory process can occur, namely non-disjunction, about which I shall talk in a moment. When a loss of one member of a chromosome pair takes place there is a premium put on duplication of the now haploid ("monosomic") chromosome. This produces homozygosis for one whole chromosome, but all the rest remains heterozygous.

Luria: Would you think that there is a premium on further losses, or that there may be in such lines a mechanism that continues to favour losses?

Brenner: Could they be clonal?

Pontecorvo: They could be both, i.e. accidental losses selected and further losses favoured by the same cause which produced the first loss. My guess is that the most common situation is simply one of selection, and not that of some permanent damage in the cell which is transmitted in the cell lineage and goes on producing chromosome losses. A third possibility would be that the imbalance, resulting from one accidental loss, favours further losses.

Lederberg: It should be stressed that you are culturing an organism in a nearly normal habitat, corresponding to the one in which its chromosome complement has evolved. The same consideration does not necessarily apply equally to tissue culture, or to transplanted

Eagle: Not too accurately—anything between pH 7 and 7·8 gives a reasonably good result. In cloning experiments, pH can be rigorously controlled by placing the open Petri dishes in a CO_2 incubator in which the pH is determined by the bicarbonate concentration of the medium, and the partial pressure of CO_2 in the gas. One should not attempt to do experiments in which pH would be a factor, in stationary and stoppered cultures, because of the wide variation (even within a 24-hour period) of the pH in a closed vessel in which the cells are rapidly elaborating lactic acid.

Harris: Is it conceivable that with really accurate control here you may not get some of these peculiar phenomena?

Eagle: It is conceivable but rather unlikely.

Monroy: In connexion with the possibility of obtaining differentiation in stabilized cultures, it seems pertinent to mention here the experiments of Niu who has been able to induce differentiation of neural and non-neural structures from explants of embryonic ectoderm under the influence of ribonucleoproteins from thymus. Although these experiments have been carried out on primary cultures, it would be worth while checking the possibilities of the method on stabilized cultures.

Ingram: Dr. Eagle, what is known about the survival of the haemoglobin-forming cells in bone marrow cultures?

Eagle: There are a number of so-called bone marrow cultures.

Ingram: Do any of them make haemoglobin?

Eagle: None, as far as I know; and in none of them can the nature of the cell be identified.

Luria: Another aspect of genetical study on cells in tissue culture is one which we may call infected heredity. We have heard earlier of two possible types of changes that may manifest themselves in cultured cell lines: mutational changes, and physiological changes analogous to enzymic adaptation or release of repressions. We must consider next the possibility of transductional changes, i.e. of genetical elements coming from the outside into cells and altering the genetic complement. Unfortunately, for the time being there is very little known about such phenomena in either human or other mammalian cells.

We should consider two types of infective heredity: the transfer of subcellular genetic fragments from cell to cell (which might even occur normally in metazoa), and the transfers mediated by viruses, which we consider as specialized vehicles for the transfer of certain specialized genetic elements. Let us use bacteria as a model. We have in bacteria a number of phenomena of infective heredity. First, the DNA genetical elements, which presumably form the bacterial chromosomes, can be transferred by means of cell extracts in

suppose that each cell included a catalogue of forbidden responses in its antibody-forming mechanisms.*

Ceppellini: But a fraction of juvenile cells is always present?

Lederberg: Yes. Sir Macfarlane has, however, suggested that most, if not all, the diversification takes place in prenatal life. I do not feel this is a necessary condition of the theory, especially as Smith's and Mitchison's work indicates that tolerance can also decay in adult life if an antigen is allowed to dissipate.

Burnet: There is still a rather large difference between what you can do in embryonic life or prenatal life and what is possible in the mature animal, but I would agree with you in general.

Ingram: Perhaps your chemostat might be the circulation of a living animal?

Eagle: Yes, it could be. In general, however, this chemostat principle would have to be adapted to monolayer cultures, since it is more difficult to get cultures started in suspension than in stationary culture. This is best reflected by the fact that the minimum inoculum for a stationary culture is one cell, but the minimum inoculum for a suspension culture, even in the established lines, is of the order of 10,000 cells per ml.

Lederberg: Dr. Eagle, did you mention that you get much slower growth in cultures containing a protein level equivalent to whole serum? This might suggest that the concentration of protein in the normal environment of the cell is one of the factors limiting proliferation and favouring functional differentiation *in vivo*.

Eagle: No, if you have a good serum, then a 20 per cent and a 5 per cent serum will be equally effective. In most of our metabolic experiments we have deliberately used dialysed serum in order to get rid of the small-molecular weight components.

Kalckar: Are there unsaturated fatty acids in the plasma?

Eagle: Yes, there are, and we have looked into this, because we had previously found that in the growth of a saprophytic spirochaete, the only function of serum protein was to provide lipid bound to the protein. We did indeed try exhaustively extracted serum, and found no difference. However, this does not exclude the participation of lipid, because no method of extraction we have yet found removes all of the lipid.

Kalckar: You cannot replace it by tweens?

Eagle: No, they cannot be substituted by lipids.

Lederberg: What is least certain at present is the number and identity of mutable sites in the stem-line cell. The simplest and most vulnerable hypothesis is of a single "globulin gene" subject to

* Discussion abbreviated. The topic has been elaborated in a recent publication: Lederberg, J. (1959). *Science*, **129**, 1649.

frequent somatic mutations. In a diploid cell there would then be two loci and a single cell might make more than two species of antibody. Coons, Nossal and White have been able to find only one kind of antibody per cell; Lennox and Cohn have reported two. Whatever the reason for the discrepancy, and we might suppose successive mutations in the two chromosomes for the latter result, the facts so far do not yet disqualify a locus on a chromosome as the mutable site. If this should become untenable we might infer either that several chromosome loci are involved, perhaps corresponding to different electrophoretic classes of antibodies, or that the mutation occurs during the fabrication of the cytoplasmic ribosomes. This version would have to be supported by some degree of autonomous replication and reactivity of individual ribosomes.

Ingram: Could you propose a molecular model for the stimulation and repression of your cells by the antigen?

Lederberg: Not a detailed one. The cytotoxicity of antigens in hypersensitivity reactions is still controversial, but the destruction of cells in the homograft reaction can hardly be doubted.

Luria: Do you think it is possible that there is some primary template which, as long as it remains combined with its product, can act as a repressor of cell growth? Combination with the antigen may release the combination and also the repression.

Lederberg: Yes. The stimulation might be analogous to recent models for enzyme induction. The inducing substrate is now believed to displace a premonitory amount of enzyme from its ribosomal template, and by so displacing it allows the continued further production of more of this protein, as well perhaps of the corresponding RNA.

Luria: No experiment has yet been done on trying to get three antibody types from one cell?

Lederberg: Not as far as I know.

Burnet: Nossal has used flagellar antigens and he has refined his method considerably by concentrating on cells with the gross morphology of plasma cells. In animals immunized with three antigens he finds without exception that only one antibody is produced by any single cell. He has also under way and partially completed experiments along the lines of those of Lennox and Cohn, i.e. using sequential immunization with two antigens. He still finds only one antibody produced from any single cell tested.

Itano: Injection of protein-hapten conjugate into an animal induces production of antibodies specific to protein and antibodies specific to hapten. When a hapten such as arsanilic acid is injected without a protein carrier, antibodies against this hapten are not induced although the hapten will react with antibodies induced by

protein-hapten conjugate. How does your theory of antibody induction account for this difference in response?

Lederberg: This is a problem for any model. There are at least two possibilities: one is that arsanilic acid needs to be complexed to reach its target in the cell, or else to react effectively with that target.

Dr. Grubb has also referred here to a "globulin gene", one which governs the antigenic specificity of globulins. This is perhaps not the same gene hypothesized in my discussion as it should control the common carrier chain, not the stereospecific segment. Porter and others have split rabbit γ-globulins into a homogeneous, crystallizable fraction, and another heterogeneous fraction. The first fraction carries the antigenic specificity and thus may well correspond to Grubb's gene. It may also be the fraction which shows the common N-terminal pentapeptide of rabbit globulins though this is still conjectural. The second fraction carries the specific combining ability of the antibody and should correspond to the "stereospecific segment" of the theory.

Ceppellini: In your experiments the lymph node was stimulated by two antigens, each one carried by different particles. One must take into account the possibility that the antibody-forming cell produces only one kind of antibody because it was stimulated by one particle or molecule. It would be interesting to see what happens when one cell is stimulated by a physical entity carrying more than one antigenic specificity; for instance, one could compare the responses after stimulation with a mixture of A and B salivas or after stimulation with AB saliva (in this case the same molecule carries the A and B groupings).

Lederberg: This particular issue could be discussed at great length. But it is not obvious how it would help us to decide between instructive and elective models. Porter's heterogeneous fraction would be the antigen to use.

Grubb: Is it essential to your hypothesis that the amino acid sequence is different in different antibodies?

Lederberg: It is not really essential to elective theories but it is most consistent with other systems of nucleic control. We could retreat to a genically controlled folding pattern, but why set antibodies apart from other specific proteins unless the chemical evidence insists?

Grubb: Is there not some evidence to the contrary, i.e. that the amino acid sequence is the same?

Lederberg: Yes, there has been for rabbit globulins but this is now quite indecisive in view of Porter's recent results. The "common carrier" may be a single protein in the case of rabbit globulin, a

variety of soluble proteins in human globulin. I am not clear whether globulins from individual genotypes have been studied for their chemical constitution. But this need have nothing to do with the stereospecific segment.

Eagle: Your theory has as an essential precondition that the antigen gets into the cell.

Lederberg: I had not doubted this but the least condition is that the antigen should react with the contained antibody of the cell.

Eagle: Would you consider as a possibility a combination of this theory which you and Sir Macfarlane Burnet propose with the instructive theory in the sense that the specifically orientated clone differs from the other only in its permeability to a given protein?

Lederberg: The main question I have been trying to answer concerns the origins of the information for the variety of proteins that antibody-forming cells could make. Your suggestion should be considered as equally applicable to instructive and elective theories.

Kalmus: Have you any estimation of mutation rates?

Lederberg: This would depend on the number of antibodies that have to be accounted for. A mutation rate of one per 100 cell divisions will give some hundred million mutant varieties among the 10 billion immunologically competent cells which have been estimated to occur in the rabbit.

Kalmus: Insects which have very poor immunological responses have to meet similar chemical challenges differently, and they meet them, e.g. by undoubtedly chromosomal mutations against new insecticides in the germ cells. As it is comparatively easy to estimate insect numbers we have some idea about the size of population and mutation rates necessary for a particular resistance to develop. Mostly but not invariably the numbers seem to be fairly large and the mutation rates low.

Lederberg: I am sure that the globulin gene must have a much higher mutation rate than the haemoglobin gene, for example. The mutability of this gene is a necessary element that would have to be evolved for it to function in immunity.

Kalmus: But in fact there is a very high mutation rate?

Lederberg: Yes.

Luria: Clearly, high mutability of such a gene will have an advantage.

Lederberg: Yes.

Ingram: I share your difficulty in believing that a single amino acid sequence can assume a large number of configurations, because you are dealing with rather simple surfaces. There have been reports in the literature that people have taken preparations of gamma globulin

and have apparently transformed them into specific antibodies by heating them with antigen.

Lederberg: Would you like to comment on that, Sir Macfarlane? I wonder if the experiment could be repeated.

Burnet: This was described in some early work by Pauling. At the present time I believe that any serum globulin which is specifically reactive with a definable organic configuration can legitimately be referred to as an antibody. Undoubtedly physical changes might be induced in a globulin by heat or the like which modified its reactivity. But I do not think that is relevant to the problem of antibody production.

they could recognize horses and women. We are presented with the proteins. Now almost certainly the semantic context of a polypeptide chain of an amino acid sequence has got nothing to do with the original nucleic acid code, but it has to do with the folding of the protein, the structure of the active centres, and so on. In other words, inspection of amino acid sequences can probably tell us nothing about the code, but everything about protein structure. That is what breaking of the protein code would mean in terms of deciphering linear B. We are, however, trying to find the letter congruences between the language of an as yet unknown system, and a system the semantic context of which we do not fully understand.

Lederberg: What do you have against including specific codes for punctuation in the dictionary? Some redundancy in these codes (e.g. STOP = ALT = ARRET) might also furnish a place to put the extra guanine you find in some DNA without having to imagine basic changes in the code itself. The common —ACC termination in soluble RNA certainly looks like punctuation.

Brenner: There is no objection to this. One can certainly have codes with a specifically coded comma. For example, consider a binary code in which two of the DNA nucleotides are equivalent so that only two different kinds of letters are used. One can show that five letters will be needed to code for twenty amino acids ($2^5 = 32$) and that eight letters will be required if the code, is to be comma-less. We write ABB as a triletter comma, and delete all those five-letter sequences which contain this comma. Of the 32 combinations, 12 are eliminated, leaving exactly twenty! The ABB could correspond to the constant ACC-termination of the soluble RNA, but this would mean that the binary code must be purines or pyrimidines. This code is not to be taken seriously.

Lederberg: If we admit punctuation into the dictionary, could we also use it to convey folding information? For example, the length of the commas and spaces might influence the compactness of amino acid assembly and the degree of freedom for folding angles.

Kalow: I was interested in your ideas on the rate of protein synthesis. Does your theory permit the assumption of a variation of protein concentration from person to person, or does it imply that a change of protein structure is necessary in order to produce a change of concentration?

Brenner: No, all the suggestions apply only at the level of the template. Once there is a huge cellular apparatus built on top of this, then there is a further source of individual variation. In the mammalian organism, there are regulatory devices superimposed on the basic system. I do not believe that all these other variations affect the protein structure but, of course, they might.

haemoglobins pooled together one could, by a very sensitive technique, detect 1 per cent of change, then this would give one courage to go on and look at them individually.

Neel: It is worth emphasizing that no matter which way the analysis of 500 unselected haemoglobin specimens turns out, i.e. whether you find variability or do not find variability, the results are equally challenging.

Ingram: In this connexion may I ask Dr. Neel or one of the other geneticists whether it is possible to take the data on the rare abnormal haemoglobins that are available, and to estimate the degree of "hidden" variability in haemoglobin which one might expect in a human population?

Neel: My answer is no.

Harris: No, but there is also another strange problem. It has always struck me as very curious that the first two mutants of haemoglobin to be worked out by you, Dr. Ingram, should turn out to be exactly at the same point in the amino acid sequence. Here there is thought to be a locus controlling the formation of a polypeptide chain with a sequence of about 150 amino acids, and the first 2 mutants to be found turn out to involve exactly the same site in this. Of the other 5 or 6 which you discussed at this symposium, one has turned out to be very close to this first point and the others also appear to be grouped non-randomly. There must be some good reasons why these points in the amino acid sequences have been picked out. Admittedly it might be caused by phenomena at various levels; but one should keep this very much in the forefront of one's mind in further work.

Ingram: The tendency at the moment is to explain this by putting the blame on the natural selection of phenotype.

Harris: Admittedly for S and C there may be something in this, but when you get beyond that to some of the others, selection is largely done blindly by electrophoresis.

Neel: Dr. Crick, in a recent conversation, emphasized the possibility that many of these changes may involve the terminal polypeptide in the molecule, a polypeptide which would not be so essential to the configuration of the protein.

Ingram: This is almost certainly not true for some of them.

Neel: We were talking about the SCG polypeptide.

Lederberg: The term "species specificity" has crept into some of the discussions, particularly of immunogenetical relationships. This may have connoted that different proteins from the same organism or species are likely to have a common mark which distinguishes them from the set of proteins of another species. As far as I know this is not so, but I would ask for contradiction. On the contrary,

one would suspect that homologous proteins will show tangible resemblances regardless of species, for example the related amino acid sequences of various insulins.

Ingram: The picture is somewhat complex, but you have both similarities and differences between the haemoglobins of various species at the finger-print level in terms of peptide sequence.

Lederberg: This clearly is a locus specificity. Has the sequence of, say, haemoglobin anything in common with the sequence of any other human protein, such that one would be willing to recognize the set of proteins as having come from the same species? This is what the serologist used to mean when he talked about species specificity. Is it a real concept?

Brenner: I don't think so, because if you take the known sequences of different proteins from the same organism and look at the distribution of dipeptides, these look rather random. I think that *a priori* it is very unlikely.

Itano: The similarities in properties and function of haemoglobin and myoglobin are such as to suggest that the genes controlling the synthesis of myoglobin and the chains of haemoglobin have a common origin. It would be of interest to compare these proteins chemically for possible similarities in positions or sequences of amino acids. Particular attention could be paid to proline because of its unique rôle in peptide bond structure.

Brenner: Whale myoglobin does not look like either horse haemoglobin or human haemoglobin.

Ingram: But you can also say that horse myoglobin and horse haemoglobin show distinct differences, both by finger-printing and, for example, in cysteine content. There may be similarities elsewhere.

Kalckar: Since we are discussing the species question, it may be relevant to refer to Tuppy's investigations on cytochrome *c* [Tuppy, H. (1957). *Z. Naturf.*, 12*b*, 184]. He has identified the amino acid composition of the haemopeptide of cytochrome *c* from various species.

Smithies: Is it not the generally held view that many groups of proteins with a similar function (e.g. haemoglobins) probably arose by evolution from a single "ancestral" protein, so that one would expect to find close similarities in the structure of a particular type of protein even in different species? Evolutionary changes occurring after species differentiation would be expected to produce more or less limited interspecies differences depending on the evolutionary time at which the division into species occurred.

Montalenti: We have come to the end of this symposium. We have all enjoyed the papers and discussions, and each one of us has