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THE RELATIONSHIP OF BIOCHEMICAL AND ENZYMIC ABNORMALITIES  
OF CELLS TO RADIATION SUSCEPTIBILITY AND TO THEIR INJURY BY  
OTHER AGENTS.

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Investigation of the relationship of biochemical abnormalities in cells to their injury by various means has been continued using primaquine-type hemolysis as the model entity. Studies of the inactivation of G-6-PD by erythrocyte stromata have shown it is caused by pyridine nucleotidase activity of the stromata with consequent removal of oxidized triphosphopyridine nucleotide (TPN) found necessary to stabilize the G-6-PD in erythrocytes. Both TPN and DPN protect the G-6-PD of erythrocytes directly. Nicotinamide protects indirectly by interfering with the pyridine nucleotidase activity of the stromata. Intact cells also exhibit this activity and the mechanism may be important in vivo. The TPN was shown to be bound to G-6-PD and rose and fell concomitantly with the activity of this enzyme (NATURE, 184, 1292, 1959).

Another investigation has been development of a technique for in vitro hemolysis. Primaquine or Furofantin when added to blood and exposed to ultraviolet light between wavelengths of 300 to 400 millimicrons are converted to hemolytic degradation products. The technique is still in a preliminary state but it can be shown that the sequence of events leading to hemolysis includes the oxidation of the reduced glutathione, then a subsequent disappearance of the oxidized glutathione and the simultaneous formation of methemoglobin. These changes are found in both primaquine-sensitive and normal cells which have been washed and resuspended in saline; in whole blood, only in primaquine-sensitive cells. Some protection against this effect is given by ethylene-diamine-tetraacetic acid (EDTA). The system offers great promise because many phenomena associated with primaquine-sensitive hemolysis can be observed which cannot be shown in vivo because the cells are lysed and cannot be recovered in drawn blood. This technique reveals that sheep cells, primaquine-sensitive by test, behave like normal human cells and suggests that failure of sheep to undergo in vivo hemolysis when given primaquine parenterally is a factor of the sheep erythrocyte itself and not of a different metabolism of primaquine. The technique of gel filtration has also been developed in this laboratory for separating free and bound forms of glutathione and coenzymes as well as the degradation products of primaquine.

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This project was undertaken and conducted by Dr. Paul E. Carson to investigate the relationship of biochemical abnormalities in cells to their injury by various means, including X-radiation and injury by drugs. The model entity chosen has been primaquine-type hemolysis which occurs in individuals whose erythrocytes are naturally deficient in glucose-6-phosphate dehydrogenase activity. These cells display other abnormalities, particularly a low reduced glutathione content and a moderately decreased activity of catalase. The latter two abnormalities are further implicated in primaquine-type hemolysis because they are unstable to the action of the hemolytic degradation products of primaquine in contrast to their stability in normal erythrocytes. The primaquine-sensitive pattern of biochemical abnormalities has now been found in the erythrocytes of individuals of all races. These individuals are all susceptible to acute intravascular hemolysis which may be induced by a great variety of hemolytic agents including some dietary products, most noteworthy of which is the fava bean (favism). Probably more than 100,000,000 people are affected. Moreover, it is becoming apparent that other tissues of these people are also relatively deficient in glucose-6-phosphate dehydrogenase. This finding considerably increases the significance of the primaquine-sensitive pattern of biochemical abnormalities, particularly in relation to other diseases.

In the previous annual report, we described both activation and inactivation of glucose-6-phosphate dehydrogenase in human and sheep

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erythrocytes when they were X-radiated under various conditions. These changes did not seem likely to be due to the direct effects on the enzyme but rather on one or more co-factors which might be involved. Therefore, we undertook further study of the inactivation of glucose-6-phosphate dehydrogenase in hemolyzates of both normal and primaquine-sensitive cells which occurs on incubation with their stromata but not in the absence of stromata. This inactivation is specific, related enzymes such as glutathione reductase and 6-phosphogluconic dehydrogenase retaining activity. Investigations by Kirkman of purified glucose-6-phosphate dehydrogenase from normal and primaquine-sensitive erythrocytes has revealed an unusual protective effect by oxidized triphosphopyridine nucleotide (TPN) which stabilized the enzyme out of proportion to the protective effect of other compounds. In hemolyzates, we found that TPN in small concentrations, for example,  $10^{-5}$  M, would protect glucose-6-phosphate dehydrogenase against inactivation by stromata. Nicotinamide, known to protect against pyridine nucleotidase activities, also protected the enzyme, although much higher concentrations are required ( $10^{-3}$  -  $10^{-2}$  M). The related co-enzyme, diphosphopyridine nucleotide (DPN) also provided protection. When the stromata were removed and the stroma-free hemolyzates treated with activated charcoal (Norit) to remove the pyridine nucleotides, inactivation of glucose-6-phosphate dehydrogenase also resulted on incubation. In these stroma-free

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hemolyzates, both TPN and DPN offered protection, complete in the case of TPN, partial for DPN. However, nicotinamide no longer protected, indicating that its effect was against the stromal pyridine nucleotidase activity, whereas the TPN and DPN protected the enzyme directly. Contamination of DPN by small amounts of TPN was ruled out by suitable control reactions. Since DPN does not protect purified enzymes, this may be indirect evidence for the enzymatic conversion of DPN to TPN in hemolyzates.

Simultaneously, investigation of the non-dialyzable TPN revealed that it rose and fell concomitantly with the activity of glucose-6-phosphate dehydrogenase and appeared, in fact, to be bound to it. Neither glutathione reductase nor 6-phosphogluconic dehydrogenase required this co-enzyme for stability nor did they bind it. Evidence was also obtained showing that the intact erythrocytes could inactivate glucose-6-phosphate dehydrogenase in isotonic hemolyzates. Furthermore, in primaquine-sensitive erythrocytes, evidence was obtained which suggested that the G-6-PD could also be inactivated within the intact cell. These processes are considered to offer one key to elucidation of the mechanism of primaquine-sensitive hemolysis and also of cellular aging. A paper based on this work was published during the year of this report in NATURE (184, 1292, 1959) and is enclosed. To date, no other substances have been found which consistently protect this enzymatic activity, either directly or indirectly. These include related compounds such as nicotinic acid, isonicotinic acid,

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isonicotinic acid hydrazide and 6-amino-nicotinamide. Ethylenediamine-tetraacetic acid (EDTA) has given inconsistent protection. Further studies of this phenomenon are being continued, both in connection with injury by X-radiation and in relation to an activating factor also found in erythrocyte stromata, recently described in Israel.

In addition to carrying on further studies concerned with the above discoveries, a considerable portion of the year has been devoted to developing new methods and techniques which offer a much broader basis for our investigations. Two of these are most exciting. One is the development of an in vitro system for primaquine-type hemolysis, utilizing instead of X-radiation, ultraviolet radiation. Primaquine is added to blood or red cell suspensions in pyrex tubes which are then submitted to ultraviolet radiation through filters allowing light of wave lengths from 300 to 400 millimicra. to pass. The ultraviolet effect is to degrade the primaquine to its hemolytic products but not to directly affect the sulfhydryl groups of the blood. The degraded primaquine can then exert its hemolytic effect and the result is a very sensitive and quantitative system for following the changes induced in the various products and pathways prior to hemolysis. These changes cannot be observed in vivo because the cells are lysed and cannot be recovered in drawn venous blood. Fura-dantin can also be used instead of primaquine in this system. Already, we have been able to show that the sequence of events includes the oxidation of the reduced glutathione prior to its disappearance and the

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simultaneous formation of methemoglobin. Although these changes have been suspected, they have not before been simultaneously demonstrable in primaquine-sensitive blood. Protection against changes induced by this in vitro technique have thus far been confined to studies utilizing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant instead of heparin as well as adding EDTA to the blood or red cell suspensions. This substance does not completely protect but gives partial protection, partly by preventing as great a fall in the reduced glutathione content and partly by protecting against the disappearance of the oxidized glutathione formed. Cation exchange is known to be changed prior to hemolysis induced by X-radiation and must be of significance in primaquine-sensitive hemolysis, also.

These studies have suggested that in vivo protection might, perhaps, be offered by substances which would alter the coenzyme levels of the red cells either directly or indirectly by altering tryptophan metabolism. To date, in cooperation with the studies being carried on at Stateville, partial protection against in vivo hemolysis has been found by using intravenous EDTA or long-term dosages of nicotinic acid. Nicotinamide does not protect. These results, of course, are preliminary and may fail to be confirmed.

In connection with the in vitro system for primaquine-type hemolysis it is important to recall that, as indicated in last year's report, sheep cells are positive by test for primaquine-sensitivity but sheep did

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not hemolyze when given primaquine in vivo parenterally. Attempts to cause intravascular hemolysis in sheep have been repeated with primaquine in dosages as high as 240 mg. per day and with Hykinone and with dimercaprol (BAL). The question arose as to whether the failure of sheep to undergo hemolysis was due to its metabolizing these drugs in a different way or because the sheep cells, themselves, are resistant. By use of the in vitro system, it has been possible to show that the sheep cells, themselves, are resistant to hemolysis and makes their continued study of more significance than we had previously thought. The in vitro system also offers an approach to the study of many other related processes. These include changes in glutathione, hemoglobin, enzymatic activities and co-factors.

The second method of significance developed during the past year requires a new compound known as Sephadex, a cross-linked dextran which is used in columns for a process called gel filtration. The substance accomplishes separations on the basis of molecular size rather than by ion exchange. In practical use, for example, protein containing solutions such as hemolysates or plasma are essentially dialyzed in the column because the large protein molecules pass through the column first, followed by the free small molecules. In effect, this technique offers a quick way of dialysis. However, beyond this, it is adaptable to batch procedures for comparing by the use of several columns simultaneously, several parameters of the red cell sensitivity to hemolysis. Among these are the quantitation

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of free and bound coenzymes in relation to the enzymatic activities of glucose-6-phosphate dehydrogenase and glutathione reductase, the comparative state of reduced and oxidized glutathione in hemolyzates from normal and primaquine-sensitive red cells and the binding to serum or plasma proteins of degradation products of primaquine. The first study is a continuation of the problem described above. The second is particularly important to help elucidate the reason for the instability of glutathione in the whole blood of primaquine-sensitive individuals as well as to delineate the apparent binding of a fraction of both reduced and oxidized glutathione to either hemoglobin or its oxidation products. Both of these problems, when enough information has been obtained, will be re-evaluated after X-radiation. The third study rests on evidence obtained by the in vitro ultraviolet radiation technique that primaquine itself which is only relatively injurious to the primaquine-sensitive red cells in vitro is not, apparently, bound to plasma proteins. However, the hemolytic degradation products formed during the radiation are, apparently, bound to the plasma proteins. This binding may be necessary for these products to exert their hemolytic action or, on the other hand, may be a means of protection against hemolysis. If specific agents to bind these products in the circulating blood could be found, the hemolytic action could be divorced from the antimalarial action in the tissues.

These combinations of techniques, together with the use of more standard procedures including spectrophotofluorometry for coenzyme

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determinations, spectrophotometry and radioisotope tracer techniques, are enabling us to delineate quantitatively the sequence of events which occurs in a primaquine-type hemolysis, and to undertake comparisons to other forms of hemolysis both in vitro and in vivo. Investigation of alterations in metabolic pathways occurring as an effect of the various injurious processes also become more feasible. A rational basis for testing the protective effect of other agents of induced alterations in metabolic pathways is now possible. Moreover, an investigation initiated in this laboratory by Dr. Walter Long is being undertaken to relate the changes occurring in other disease processes to the activities of the enzymes which have been under study in this laboratory. Special reference is being given to changes in activities of glutathione reductase, which we have previously suggested may be a pivotal reaction between the glycolytic and oxidative pathways of carbohydrate metabolism.

Finally, a survey of patients with primaquine-sensitivity to determine if they have a higher incidence of cataracts has been undertaken in cooperation with Dr. Frank Newell, Professor of Ophthalmology.

During the past year, Doctors Carson and Alving were invited to speak at a Symposium at the Teaching Institute for the American Society of Pharmacology and Experimental Therapeutics which was held in Miami on August 31, 1959 and, more recently, Dr. Carson was invited to speak on Glucose-6-Phosphate Dehydrogenase in Hemolytic Anemia at the Symposium on Lesions of Carbohydrate Metabolism held by the

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American Society of Biological Chemists during the Federation meetings in Chicago. The latter invitation also represents an invited manuscript which will appear in the Symposium Issue of the Federation Proceedings during the coming year.

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