contained no C^{14}. A possible loss by excretion through the gastrointestinal tract cannot be excluded.

We believe that the galactose was not only metabolized to glucosiduronic acid but that most of the remaining 200 mg of galactose was generally metabolized. This belief is partly based on an experiment in which only 1.5 mg of galactose-1-C^{14} (undiluted) was administered. In the afore-mentioned experiment, it was established that the galactosemic organism is able to metabolize 30 mg of galactose to glucosiduronic acid. One would therefore expect that if the latter pathway were the only one in operation, a minute amount of galactose, such as the 1.5 mg of C^{14}-labeled galactose that was administered in a separate experiment, would readily be completely converted to glucosiduronic acid. However, the experiment showed that only 6.5 percent of this amount appeared as glucosiduronic acid. The most reasonable assumption is that the rest went through the pathway of glucose-6-phosphate and hence escaped detection by high isotope dilution. These considerations probably also apply for the experiment in which 1 g of galactose was administered.

The special value of the isolation and analysis of the C^{14}-labeled menthyl glucosiduronic acid is manifold. (i) It demonstrates directly that galactose can definitely be metabolized in the galactosemic organism. (ii) It poses a question as to whether the block of the galactose-1-phosphate uridyl transferase is complete, for it is known from earlier studies that conjugated glucuronic acid arises from hexoses through the uridine nucleotides (*7*).

That the normal pathway, or a very closely related accessory one (compare *14*), was in operation in this case is also supported by the fact that the distribution of labeled carbon in the glucuronic acid moiety was identical with that of the galactose administered—that is, it was confined to carbon atom 1 (*15*).

A normal adult person is able to metabolize 20 to 25 g of galactose within 12 hours (*16*), whereas a galactosemic person is able to metabolize 150 to 200 mg at the most. This means that a galactosemic subject has retained only about 1 percent of the capacity of a normal person with respect to galactose metabolism. This is a somewhat lower than, but probably in the order of magnitude as, that found *in vitro* by enzymatic assays on liver tissue obtained from the same patient (*6*). It was estimated that less than 4 to 8 percent of the normal activity of the galactose-1-phosphate uridyl transferase was retained in the galactosemic liver. Alternatively, the activity could be attributed to an incorporation enzyme related to galactose-1-phosphate uridyl transferase. The red cells from the same subject showed no detectable amounts of galactose-1-phosphate uridyl transferase.

The values obtained from *in vitro* and *in vivo* studies definitely fall into the same order of magnitude. The *in vivo* method is for this particular purpose as sensitive as the *in vitro* technique. Both methods may give the approximate residual galactose-1-phosphate uridyl transferase present in the galactosemic subject. However, it is quite naturally not possible to rule out the existence of a closely related accessory exchange mechanism involving galactose-1-phosphate, which is replacing the normal pathway in congenital galactosemia.

FRANK EISENBERG, JR.
KURT J. ISSELBACHER*
H. M. KALCKAR

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

References and Notes

1. H. H. Mason and M. C. Turner, *Am. J. Diseases Children* 50, 359 (1935); E. H. Townsend, Jr., H. H. Mason, P. S. Strong, *Pediatrics*, 7, 760 (1951); D. J. Bell, *Biochem. J. (London)* 30, 1612 (1936); E. Bruck and S. Rapoport, *Am. J. Diseases Children* 70, 267 (1945).
2. V. Schwartz *et al.*, *Biochem. J. (London)* 62, 40 (1956).
3. H. M. Kalckar, E. P. Anderson, K. J. Isselbacher, *Proc. Natl. Acad. Sci. U.S.* 42, 49 (1956).
4. K. J. Isselbacher *et al.*, *Science* 123, 635 (1956).
5. H. M. Kalckar, E. P. Anderson, K. J. Isselbacher, *Biochim. et Biophys. Acta* 20, 262 (1956).
6. E. P. Anderson, H. M. Kalckar, K. J. Isselbacher, *Science*, this issue.
7. G. J. Dutton and I. D. E. Storey, *Biochem. J. (London)* 57, 275 (1954); J. L. Strominger *et al.*, *J. Am. Chem. Soc.* 76, 641 (1954).
8. Galactose-1-C^{14} was obtained through the courtesy of H. Isbell, National Bureau of Standards.
9. R. T. Williams, *Biochem. J. (London)* 34, 272 (1940).
10. F. Eisenberg, Jr., and S. Gurin, *J. Biol. Chem.* 195, 317 (1952).
11. Z. Dische, *ibid.* 204, 989 (1953).
12. N. Nelson, *ibid.* 153, 375 (1944).
13. E. R. Froesch and A. E. Renold, *Diabetes* 5, 1 (1956).
14. E. E. B. Smith, A. Munch-Petersen, G. Mills, *Nature* 172, 1061 (1953).
15. F. Eisenberg, Jr., and K. J. Isselbacher, unpublished.
16. H. J. Deuel, *Physiol. Revs.* 16, 173 (1936).

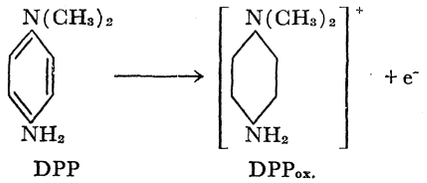
* Present address: Massachusetts General Hospital, Boston, Mass.

14 November 1956

Oxidation of N,N-Dimethyl-p-phenylenediamine by Serum from Patients with Mental Disease

In investigations carried out at the Långbro Mental Hospital in 1955, it was found that fresh blood serum obtained from patients with mental disease, including schizophrenic, manic depressive, and senile psychoses (*S*-serums) had the capacity to oxidize N,N-dimethyl-*p*-phenylenediamine (DPP) more rapidly than fresh serum obtained from healthy control subjects (*N*-serums). Further investigations to determine the incidence of this reaction in nonmental diseases and to explore the possibility of using it as a diagnostic test for mental disease are in progress. This report describes the biochemical background that underlies this reaction (*1*).

Mild oxidation of DPP leads to the formation of a free radical of a semiquinone type (see for example, *2*):



DPP DPP$_{ox.}$

This substance has an absorption maximum at 552 mμ and is known as Wurster's red.

The addition of DPP (*3*) to fresh serum from healthy control subjects is followed by a lag period of approximately 5 minutes, during which there is but slight increase in the extinction at 552 mμ. A sudden acceleration in color development then ensues, with a linear increase in extinction. In contrast, the lag period in development of the red color is much shorter or absent with fresh *S*-serum (Fig. 1).

Prolonged exposure to room air, gentle aeration, or dialysis against 0.9-percent NaCl increases the capacity of *N*-serum to oxidize DPP so that it equals or approaches the rate characteristic of *S*-serum. This suggests that one or several reducing substances of low molecular weight which are present in *N*-serum are quantitatively diminished or absent from *S*-serum.

The DPP-oxidizing substance in both serums is heat labile and not dialyzable. It therefore appears to be a catalytically active protein.

The ability to oxidize DPP disappears when serum is dialyzed against a citrate-phosphate buffer of *p*H 3. Following removal of buffer ions by dialysis against 0.9-percent NaCl, this activity can be restored by addition of a low concentration of Cu^{++} ions but not by Fe^{+++} ions. This finding suggested that ceruloplasmin, a serum oxidase containing copper, which was first isolated by Holmberg and Laurell (*4*), might be involved. Ceruloplasmin constitutes the copper-binding protein of normal serum. It contains about 95 percent of the total serum copper (*5*) and is also capable of catalyzing the oxidation of *p*-phenylenediamine.

Evidence suggesting that catalytic oxidation of DPP by serum is attributable to ceruloplasmin was provided by finding that this activity is precipitated with $(NH_4)_2SO_4$ in the same fraction as is ceruloplasmin. Furthermore, DPP oxi-

dation by serum resembles ceruloplasmin activity in that it is strongly inhibited by NaN_3 but not by NaF, NH_4-oxalate, or cystine (inhibitor concentration about 1 mg/ml). A concentration of $2 \times 10^{-4} M$ NaN_3 produced 67-percent inhibition of serum DPP oxidase activity at *p*H 6.6, a value similar to that reported for pure ceruloplasmin with *p*-phenylenediamine as substrate (*6*). Other enzyme inhibitors such as Na-diethyldithiocarbamate, KCN, and cysteine could not be tested by this method because they decolorize DPP_{ox}.

Finally, addition of ceruloplasmin (*7*) to serum showed that DPP actually is a substrate for this enzyme. Thus the catalytically active protein responsible for DPP oxidation by serum is probably identical with ceruloplasmin.

Elevated serum copper levels have been found in schizophrenia (*8*), and recently Ozek (*9*) has shown that this increase in serum copper is attributable to an abnormally high ceruloplasmin content. Confirmation of elevated ceruloplasmin activities in *S*-serums has been obtained using DPP as substrate.

As has already been pointed out, the reducing substance (or substances) predominantly present in fresh *N*-serum is rapidly autooxidized and is dialyzable. The addition of organic Hg-halides or N-ethyl-maleiimide to *N*-serum does not effect the capacity of the serum-reducing substances to decolorize DPP_{ox}. However, the addition of physiological amounts of ascorbic acid to dialyzed *N*-serum restored its capacity to reduce DPP_{ox} (Fig. 2). It thus appears that ascorbic acid, which is present in serum in amounts of about 1 to 25 μg/ml, is responsible for at least a part of the reducing capacity of serum. It is further known that the serum ascorbic acid content is low in schizophrenia (10).

To determine whether ascorbic acid is the sole or the predominant reducing substance in serum, the effect of the addition of various amounts of ascorbic acid

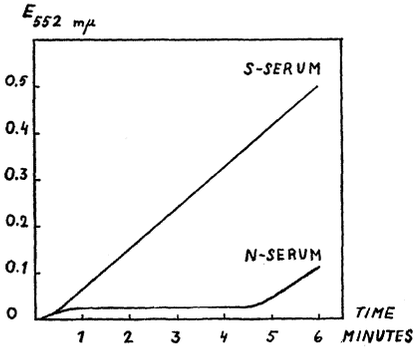


Fig. 1. Typical oxidation curves for DPP in serum; *S*-serum was from an acute schizophrenic; *N*-serum was from a healthy person; 1.5 ml of serum and 1.5 ml of 0.1-percent DPP · 2HCl in distilled water; the blank contained 1.5 ml of serum and 1.5 ml of distilled water.

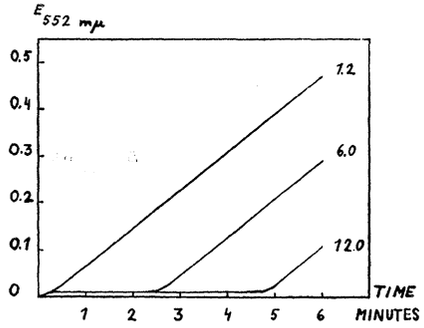


Fig. 2. Addition of ascorbic acid to dialyzed serum; 1.4 ml of dialized serum, x ml of ascorbic acid stabilized in albumin solution, $(0.1 - x)$ ml of distilled water, and 1.5 ml of 0.1-percent DPP · 2HCl in distilled water. The figures on the graph denote the added ascorbic acid (x) in micrograms per milliliter.

to dialyzed serum was investigated. Figure 2 shows that the time T during which DPP is maintained in the reduced state, as indicated by the failure of the extinction at 552 mμ to increase, is lengthened by increasing the concentration of ascorbic acid. In each instance, T is approximately proportional to the ascorbic acid content.

However, in addition to the effect of ascorbic acid, it is to be expected that T would vary inversely with the DPP oxidase activity of individual serums. The ceruloplasmin activity is indicated by the slope v of the curves in Figs. 1 and 2. If ascorbic acid is the only reducing substance playing an effective role in this reaction, a quantitative estimate of its concentration in serum can be obtained by calculation from the ceruloplasmin activity as indicated by the slope v and the measured T-value according to the following equation:

$$[\text{Ascorbic acid}] = kvT \qquad (1)$$

where k is a constant.

Variable amounts of ascorbic acid were added to a number of dialyzed serums having different ceruloplasmin acitivties, and T and v were measured. Equation 1 was found to be valid, and a k-value of 30 was found at $21 \pm 2°C$ (*11*).

It is pertinent to discuss the role of physically dissolved oxygen in this reaction and whether it is present in sufficient amounts to support the oxidation of DPP during the period of the test—that is, 5 to 6 minutes. From Eq. 1 it follows that the rate of oxidation of ascorbic acid in *N*-serum ($v \approx 0.08$) is about 2.4 μg/min (1.4×10^{-2} μmole/min), which is equivalent to 2.8×10^{-2} μmoles of DPP per minute, since 1 mole of ascorbic acid reduces 2 moles of DPP_{ox}. The concentration of dissolved oxygen in venous serum (plasma) is about 60μ*M*. In the test, 1.5 ml of serum is used, thus containing about 9×10^{-2} μmoles of O_2. It is thus apparent that the physically dissolved oxygen in serum is sufficient to account for the oxidation of DPP for about 13 minutes at the aforementioned rate, since each mole of O_2 is capable of oxidizing 4 moles of DPP. Actually, the capacity of the reaction mixture to oxidize DPP is greater, because the distilled water used contains an appreciable amount of dissolved O_2.

The ascorbic acid concentrations calculated by measuring T and v and inserting the values in Eq. 1 were compared with the ascorbic acid concentrations found by chemical analysis (*12*) of 16 different serums from both mentally diseased and healthy subjects. Good agreement between the calculated and measured values was obtained in all cases investigated, and it appears that ascorbic acid accounts for at least 95 percent of the substance in serum that is capable of reducing DPP_{ox}.

This method affords a simple means for determining the approximate concentration of ascorbic acid in serum, which should be adequate for most clinical purposes. The error is about ± 1 μg ascorbic acid per milliliter of serum. However, T is short and more difficult to measure when the ascorbic acid concentration is very low.

In those serums investigated, the ceruloplasmin activity was usually proportional to the copper content. However, rather large deviations were sometimes observed, probably depending on protein —SH— groups present in varying concentrations in different serums. These were shown to inhibit the ceruloplasmin activity, because the inhibition could be prevented by the addition of organic Hg-halides to serum.

It may be concluded from these studies that the difference between *N*-serum and *S*-serum in their capacities to oxidize DPP is dependent mainly on the fact that the ceruloplasmin activity is higher and the ascorbic acid concentration lower in *S*-serum than in *N*-serum.

The possibility of using this color reaction as a diagnostic test in mental disease is now being investigated. However, it cannot be regarded as specific for mental disease, since there are other conditions in which serum ascorbic acid concentration may be lower and serum ceruloplasmin activity higher than normal (for example, liver disease, pregnancy).

STIG AKERFELDT

Nobel Medical Institute, Stockholm, Sweden

References and Notes

1. My sincere thanks are due to Hugo Theorell for his kind interest in this work and for the hospitality of his laboratory, to Erik Goldkuhl for suggesting this field of investigation and for supplying serums, and to George Ludwig for aiding with the manuscript. This work was supported by a grant from the Swedish Medical Research Council.

2. L. Michaelis and S. Granick, *J. Am. Chem. Soc.* 65, 1747 (1943).

3. DPP is most conveniently added as the dihydrochloride salt (DPP · 2HCl), which is quite stable when dry and only slightly autooxidized in solution.
4. C. G. Holmberg and C.-B. Laurell, *Acta Chem. Scand.* 2, 550 (1948).
5. C.-B. Laurell, Kongress der Europäischen Gesellschaft für Hämatologi, Freiburg (1955).
6. C. G. Holmberg and C.-B. Laurell, *Acta Chem. Scand.* 5, 476 (1951).
7. The ceruloplasmin was kindly supplied by C.-B. Laurell.
8. L. Heilmeier, W. Keiderling, C. Struwe, *Kupfer und Eisen als Körpereigene Wirkstoffe und ihre Bedeutung beim Krankheitsgeschehen* (Jena, 1941); W. Breuner and A. Breier, *Z. Kinderheilk.* 66, 620 (1949); A. Bischoff, *Monatsschr. Psychiat. Neurol.* 124, 211 (1952).
9. M. Ozek, Kongress der Gesellschaft Deutscher Neurologen und Psychiater, Hamburg (1955).
10. P. Nilsson, *Nord. Med.* 1, 600 (1939); Y. Monauni, *Klin. Wochschr.* 29, 1036 (1937).
11. Ascorbic acid concentration is expressed in micrograms per milliliter when 1.5 ml of 0.1-percent DPP · 2HCl in distilled water is added to 1.5 ml of serum and v is measured in a 1-cm cuvette and T is expressed in minutes.
12. O. H. Lowry, J. A. Lopez, O. H. Bessey, *J. Biol. Chem.* 160, 609 (1945).

17 December 1956

Penicillin-Induced Lysis of Escherichia coli

This communication reports some results of studies the objective of which was the elucidation of the mechanism of the bactericidal action of penicillin. Those of our findings which are coincidental to the observations of Liebermeister and Kellenberger (*1*) and of Lederberg (*2*) on the penicillin-induced emergence of bacterial protoplasts were established independently.

While several metabolic processes in microorganisms are known to be influenced by penicillin, none of them has been shown conclusively to be the site of the primary action of the antibiotic, and thus, to be originally responsible for the antibacterial action of the drug (*3*). The present report is concerned with the lysis of *Escherichia coli* induced by penicillin, a phenomenon that points to profound disturbances of cell-wall functions.

Escherichia coli strain B was used as the test organism (*4*). The method for spectrophotometric assay of antibiotic action has been described elsewhere (*5*). Figure 1 shows the lysis of *E. coli* B under the influence of 100 units of penicillin per milliliter; the rates of lysis were approximately dependent on penicillin concentrations over a range from 12.5 to 500 units/ml.

Penicillin-induced lysis of *E. coli* occurred only in a nutritional environment that was capable of supporting the growth of the bacteria. Logarithmic cultures of *E. coli* which were washed free of growth medium and then resuspended in fresh media devoid of sources of carbon or nitrogen did not undergo lysis in the presence of penicillin, while bacteria that were resuspended in complete growth medium lysed as usual.

When 100 units of penicillin per milliliter was added to logarithmic mass cultures of *E. coli* B and vigorous aeration was continued, the cultures soon began to foam, and masses of macroscopic, long strands that gave the impression of highly polymerized material appeared. Strands collected by low-speed centrifugation were readily dissolved in 0.5*N* NaOH to form viscous solutions, while some residue remained when the strands were extracted with 5 percent perchloric acid for 30 minutes at 70°C. The ultraviolet absorption spectra of such perchloric acid extracts closely resembled those of nucleic acids. Chemical analysis indicated that about 15 percent of the total nitrogen of the strands went into the perchloric acid extracts. These extracts also contained quantities of pentose and deoxypentose, which suggested the presence of ribonucleic and deoxyribonucleic acids in a ratio of 3.5 to 1.

Another method of lysing bacteria, depolymerization of the cell walls by lysozyme, exposes the bacterial protoplasts (*6*), which, in an unfavorable osmotic environment, are disrupted and yield the cytoplasmic constituents in an alkali-soluble form that contains highly polymerized deoxyribonucleic acid (*7*).

In order to investigate the possibility that protoplasts might become demonstrable also as the result of penicillin action, sucrose was added to logarithmic cultures of *E. coli* B to give molar concentrations of 0.32 or 0.48. Penicillin, to a concentration of 50 units/ml, was added 30 minutes later. Incubation was continued without aeration or mechanical agitation, and samples were taken at 30-minute intervals for spectrophotometric readings and examination under the phase-contrast microscope. Figure 1 shows the time course of penicillin action in the presence of 0.32*M* and 0.48*M* sucrose.

Observation under the phase-contrast microscope (Fig. 2) revealed the following sequence of events: the bacterial rods produced central or terminal globular extrusions that increased in size while the bacterial cell walls became correspondingly empty of cytoplasm. Later, the globes either separated from the cell walls or retained parts of them attached,

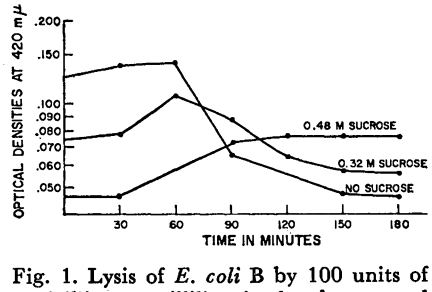


Fig. 1. Lysis of *E. coli* B by 100 units of penicillin per milliliter in the absence and presence of sucrose.

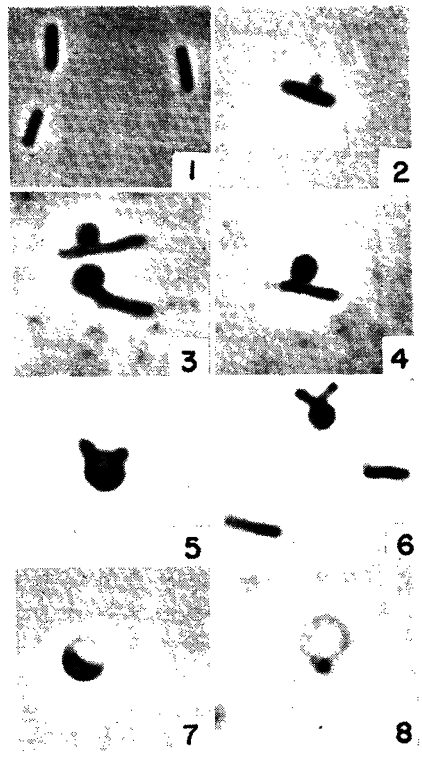


Fig. 2. Sequential phases of penicillin-induced lysis of *E. coli* B: 1, Bacteria immediately after addition of penicillin; 2, 3, and 4, emergence of globular extrusions; 5 and 6, "rabbit ear" forms; 7, partially vacuolized globular structure; 8, "ghost" form of a cytoplasmic membrane.

giving a typical "rabbit-ear" appearance. Finally, the globular structures underwent partial vacuolization, showing many crescent-shaped forms; eventually they released their entire content, leaving as formed elements only circular "ghosts" that probably represented empty cytoplasmic membranes.

The emergence from bacteria of spherical structures, called "large bodies," has been the subject of a literature more extensive than conclusive. The present morphological observations resemble those of Liebermeister and Kellenberger (*1*) concerning the action of penicillin on *Proteus vulgaris* in liquid cultures; these two authors also have emphasized the similarity between "large bodies" and bacterial protoplasts. For *E. coli*, this idea has been further expanded by Lederberg (*2*).

The present findings on the lysis of *E. coli* by penicillin are consistent with the following interpretation: the drug induces a metabolic change that affects the integrity of the bacterial cell walls in such a way that the content of the bacteria, enclosed in a cytoplasmic membrane, extrudes as a globular structure. Without protection against osmotic and mechanical disruption, the globes disintegrate and release the cytoplasmic material. In the presence of appropriate