E. coli K 13 (*4*) and have found actinomycin D to be highly effective in suppressing the synthesis of RNA by these preparations. Cells were grown at 37°C either in minimal medium (medium A of Torrain, *5*) with 0.3 percent glycerol, or in Tryptone medium (*6*) with 0.1 percent glucose. Logarithmically growing cells (50 ml) were centrifuged and suspended in 1 ml of tris-sucrose (0.25*M* tris, *p*H 8.1, and 0.3*M* sucrose). Freshly dissolved lysozyme (60 μg) and ethylenediaminetetraacetic acid, *p*H 8 (6 μmole) were added (procedure modified from that of Mahler and Fraser, *7*). After 10 minutes at room temperature the protoplasts were resuspended in their original medium supplemented with 20 percent sucrose and 0.1 percent bovine serum albumin. The suspension of protoplasts was incubated at 37°C, and RNA synthesis was measured by determining the incorporation of C^{14}-uracil into an RNA fraction prepared according to the method of Schmidt and Thannhauser (*8*). To determine the effect of actinomycin on RNA synthesis, C^{14}-uracil was added 3 minutes after the addition of various doses of the antibiotic, and the protoplasts were incubated for 20 minutes. The relative amount of RNA synthesized during this period was measured; Fig. 1 shows that 0.2 μg of actinomycin per milliliter inhibited RNA synthesis almost completely.

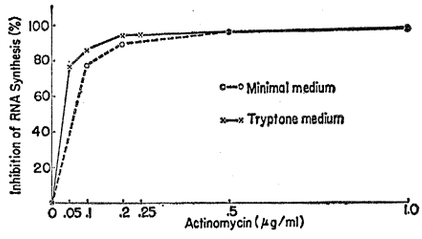

Fig. 1. Aliquots of protoplast suspension incubated for 20 minutes with various concentrations of actinomycin and with C^{14}-uracil (0.8 μc/ml, 0.3 μg/ml) were precipitated in the cold with $HClO_4$ (0.25*N*). The precipitate was washed three times with 0.25*N* $HClO_4$, then with ethanol-ether (1:3) and ether, and it was hydrolized in 0.5*N* KOH at 37°C for 18 hours. After neutralisation with $HClO_4$ the optical density of the supernatant was determined at 260 mμ in a Zeiss spectrophotometer, and the radioactivity of an aliquot measured in a Packard spectrometer. The ratio of the number of counts per minute to the optical density at 260 mμ for the untreated sample was considered 100 percent RNA synthesis. Each incorporation experiment was performed in duplicate.

The inhibition of DNA-dependant RNA synthesis in *E. coli* by actinomycin should prove to be a useful tool in the elucidation of such critical problems as the fate of phage informational RNA.

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5 March 1963

Hydrogen-Aluminum Clays: A Third Buffer Range Appearing in Potentiometric Titration

Abstract. *Wyoming montmorillonite (bentonite, particles 2 to 0.2 μ in diameter) treated with hydrogen-ion-saturated resin shows, on titration in 1N KNO_3 with NaOH by a continuously recording instrument, a third buffer range between* p*H 5.5 and 7.6 in addition to the first range where exchangeable hydronium is neutralized and the second range where a reaction with exchangeable aluminohexahydronium, $Al(OH_2)_6^{3+}$, occurs. The third range increases considerably when the hydrogen-ion-saturated clay is aged, and is attributed to basic aluminum compounds formed in the presence of negative charges of montmorillonite, comparable to "third range" buffering noted in aged, partially neutralized aluminum chloride solutions.*

Hydrogen-ion- (H) saturated montmorillonite changes to an aluminum-saturated form on aging (*1*). Among factors which influence the velocity of this transformation are temperature (*2*) and chemical composition of the clay (*3*). By this transformation, the electrostatically bonded hydronium associated with montmorillonite as a strong acid which functions in the first buffer range changes to aluminohexahydronium (*4*), which functions as a weak acid and exhibits the second buffer range, $pK_1 = 5$ (*5*). A still weaker acid group (third buffer range) on clays from soil has been indicated (*6*) by the considerable lime requirement that acid subsoils low in organic matter exhibit above the *p*H range in which monomeric aluminohexahydronium participates in exchange with KCl. This third buffer range had not so far been observed on potentiometric titration of hydrogen-aluminum clay minerals.

Recent potentiometric titration experiments with Wyoming montmorillonite (bentonite, particles 2 to 0.2 μ in diameter) treated with H-resin indicate that a very weak acid group appears as a third buffer range if the titration curve is made with a continuously recording instrument and in a fairly high concentration of electrolyte. The curves shown in Fig. 1 were obtained with a 0.5 percent suspension in 1*N* KNO_3 of montmorillonite treated with H-Amberlite IR-120, by means of a potentiograph provided with an automatic burette (Metrohm, Switzerland) and a titration rate of 0.0065 meq of NaOH per min, to 164 mg of clay. The third buffer range occurs, under these conditions, from *p*H 5.5 to 7.6 and is separated from those ranges where OH_3^+ and $Al(OH_2)_6^{3+}$, aluminohexahydronium, are neutralized. The third buffer range is detectable also after the clay is treated with potassium acetate of *p*H 5.4 instead of with H-resin.

The change of the titration curves, in particular that of the third range, has been followed during the aging of the montmorillonite suspension after treatment with H-resin. Figure 1 shows the curves after aging periods of 0, 18, and 71 hours, and 80 and 135 days, respectively.

The base consumption attributable to exchangeable hydronium and to aluminum as well as to the third range was estimated from the points of inflection of the curves and expressed as percent of total base consumption up to about *p*H 7.6 (Table 1). The percentage of exchange saturation with OH_3^+ decreased with time to almost zero; Al^{3+} first increased, then decreased. On the other hand, the third range increased throughout the aging period. The reversibility of this change in acid-group distribution was shown when the sample, aged for 135 days, was re-treated with H-resin. As the upper curve in Fig. 1 shows, the original curve was fully restored.

The continuous increase of this weak acid group (the third buffer range) during aging, concurrently with decrease of exchangeable Al^{3+}, and the complete reversibility of the reaction which occurred on acid (H-resin) treatment indicate that the proton-retaining site of the third buffer range originates by slow hydrolysis and interlayer polymerization of monomeric Al^{3+} during aging. This results in the formation of nonexchangeable hydroxy aluminum in the clay—a mechanism deduced from aluminum chemistry (*4*) and which has been extensively carried out in montmorillonite (*7*). Partially neutralized $AlCl_3$ solutions develop polymerized hydroxy aluminum ions (*8*). Layer silicate clays liberate monomeric aluminohexahydronium, $Al(OH_2)_6^{3+}$, ions during acid aging (*1, 2, 3*), and these ions hydrolyze under the concentration or pinching influence of the negative ion-exchange sites (*4, 6, 7, 9*) in the clay to form hydroxy-aluminum polymers (X) of positive charge. The positive charge arises from the excess protons of the polyaluminohexahydronium (*4*) edges, $X(OH)Al(OH_2^{0.5+})$. The third buffer range then would be attributable to the reaction of the NaOH with some of the substituted hydronium edge groups to form more nearly neutral $Al(OH)_3$, rather than with edge groups of Al(OH) or Si(OH) in layer silicates or in free silicic acid. The half-neutralization *p*H value of the third buffer range occurs at about *p*H 6.5, well below that of silicic acid ($pk = 9.5$).

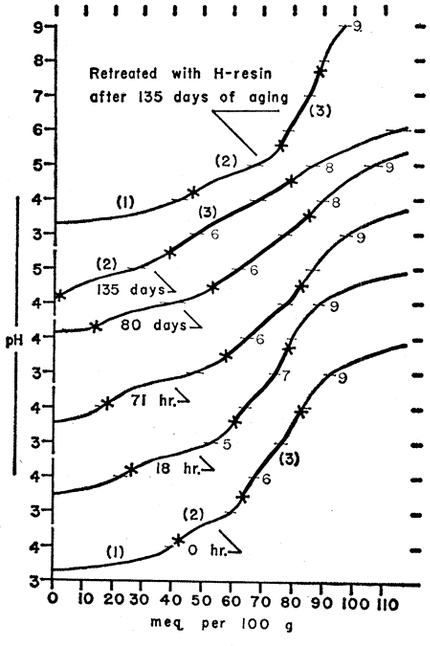

Fig. 1. NaOH titration curves of acid montmorillonite after varying periods of aging, and reversal of aging effect by H-resin treatment. Range (1), OH_3^+ neutralization; range (2), $Al(OH_2)_6^{3+}$ precipitation; range (3), hydroxy Al(+) polymer neutralization. Small numbers on curves show *p*H values.

This explanation is supported by experiments in which 0.1*N* $AlCl_3$ solutions, partly neutralized by NaOH so that the ratio of OH/Al was in the range of 1 to 2.5, were titrated in 1*N* KNO_3 with 0.01*N* NaOH after aging for 7 months. The curves show another buffer range, analogous to the third buffer range, in addition to the ordinary reaction of aluminohexahydronium with NaOH. This additional buffer range does not occur to an appreciable extent during similar rapid titration of solutions of $AlCl_3$, $Al(NO_3)_3$, or aged but not partly neutralized $AlCl_3$. The extra buffer range with solutions of $AlCl_3$ was attributed by several authors (*8*) to the neutralization of the 5/6 basic aluminum chloride $Al_2(OH)_5Cl$. The basic compound is fairly stable in dilute acids at room temperature, whereas freshly precipitated $Al(OH)_3$ is not; the basic compound dissolves in dilute acid after being heated to 75C°, and yields the original amount of Al^{3+} titratable as aluminohexahydronium.

The protons of exchangeable hydronium ions of acid clay decompose the lattice edges to liberate Al^{3+}, which on hydrolysis and polymerization liberates additional protons, in a continuing cycle (*4*). This leads to accumulation of appreciable amounts of basic aluminum polymers in the interlayer space of expansible layer silicates, as outlined in detail by Jackson (*4, 6, 9*); these polymers give rise to the third buffer range shown here, and the corresponding soil-colloid compositions and properties. The proton acceptor, or the cause of the decline in proton activity that allows the chain reaction to continue to liberate aluminum, might be the silica at the edges of the crystal structure in the clay which is converted to silicic acid gel on removal of aluminum ions (*4*) or to soluble silicic acid (*9*).

Studies with vermiculite (*10*), show that the tendency for third buffer range to appear on aging is greater in vermiculite than in montmorillonite; this could presumably be attributable to the higher surface charge density (*4, 9*) of vermiculite.

Addition of hydroxy-aluminum solutions, as mentioned above, to vermiculite yields hydroxy-aluminum polymers that are nonexchangeable from clay with KCl (*11*). The third buffer range is also exhibited (*10*) when vermiculite preparations are titrated in the same manner; the hydroxy-aluminum was only partially soluble when the vermiculite was treated again with H-resin. Both the aged vermiculite as well as that treated with hydroxy-aluminum solutions lose part of their collapsibility on treatment with potassium as estimated from X-ray diffraction data.

Table 1. Distribution of OH_3^+, Al^{3+}, and the third buffer range on montmorillonite in percentage of total base consumed after different periods of aging.

OH_3^+	Al^{3+}	Third range
	No aging	
53	30	17
	18 hours	
34	47	19
	71 hours	
25	53	22
	80 days	
12	56	32
	135 days	
0	45	55

From preliminary studies with soil clays (*10*), this third buffer range is of considerable importance even when the soil clays are freshly treated with H-resin. Since acid soil clays are usually more strongly weathered than montmorillonites from volcanic ash beds, the NaOH consumption of soil clays above *p*H 5.5 to 6 may result partially from the very weak acid groups of hydroxy-aluminum polymers formed from aluminum ions liberated during natural weathering (*12*).

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Shunt Bilirubin: Evidence for Two Components

Abstract. *Studies with C^{14}-labeled glycine and δ-aminolevulinic acid as heme-bilirubin precursors in man indicate that the early labeled or shunt bilirubin consists of two fractions. Fraction 1 requires 1 to 24 hours for maximum synthesis, is not dependent on marrow erythropoietic heme synthesis, and is possibly of anabolic origin (formed by a direct pathway from heme precursors). Fraction 2 requires 3 to 4 days for maximum production, is dependent on heme synthesis, and probably has its origin in the bone marrow, as a degradation product of red-cell heme.*

The observations of London, West, Shemin, and Rittenberg (*1*) and Gray, Neuberger, and Sneath (*2*) indicated that 10 to 20 percent of the bile pigment excreted by humans is derived from sources other than circulating red cells. This fraction was recognized by the appearance of N^{15}-labeled stercobilin within 5 to 10 days of the administration of N^{15}-glycine. This pigment, which appears much in advance of that derived from the heme of circulating red cells, has been termed the "early labeled" or "shunt fraction." It occurs at elevated levels in persons with certain diseases, among them pernicious anemia, congenital porphyria, thalassemia, refractory anemia, bleeding, and some cases of congenital hyperbilirubinemia due to primary overproduction (*3*). In these circumstances, this early labeled fraction may account for as much as 70 percent of the total bile pigment. It has been suggested that this early labeled stercobilin arises from one or more of the following sources: (i) hemoglobin of newly formed red cells destroyed shortly after formation; (ii) intracorpuscular degradation of hemoglobin in erythrocyte precursors in the marrow; (iii) heme formed in excess of globin and rapidly converted to bile pigment; (iv) other heme compounds such as myoglobin, catalase, peroxidase, or the cytochromes; (v) a direct synthetic pathway from a common precursor pool that does not require the synthesis of heme as an intermediate compound.

In a recent study reported from this laboratory, we were able to quantitate and time the appearance of this early labeled or shunt bilirubin in dogs in which a biliary fistula had been made ("bile fistula" dogs). The dogs were given glycine-2-C^{14} intravenously (*4*). In normal dogs this shunt accounted for 5 to 16 percent of a proposed common heme-bilirubin precursor pool, and labeled bilirubin appeared in the bile of all dogs within 4 to 8 hours of the injection of glycine. The formation of labeled bilirubin observed in some animals prior to the labeling of heme in the red blood cells or the marrow buffy coat (including red cell precursors) suggested that the heme in these sites was not an obligatory precursor of this bilirubin. Thus the possibility that there is a direct synthetic pathway of bilirubin from heme precursors was raised. We now report evidence that the early labeled fraction of bilirubin consists of two components which arise through different pathways.

Bilirubin was isolated from plasma by precipitation of the plasma proteins with ammonium sulfate and alcohol by a modification of the method described by Cole and Lathe (*5*). The bilirubin was then taken up in chloroform and purified by column chromatography to constant specific activity (*4*). Hemin was prepared and assayed for radioactivity (*4*). Fifty microcuries of glycine-2-C^{14} (Picker Nuclear) was given intravenously to a 112-kg man. Venous blood was drawn at intervals, and the bilirubin and hemin were isolated and assayed for radioactivity. The results are shown in Fig. 1. Radioactivity appeared in circulating heme within 24 hours and reached a plateau of 10 counts per minute per milligram on the 8th day. Labeled bilirubin was first detected at 3 hours and exhibited an initial peak at 24 hours; the activity then declined to a minimum at 48 to 60 hours, and a second peak was observed on the 3rd and 4th days. The same pattern was observed in two additional subjects.

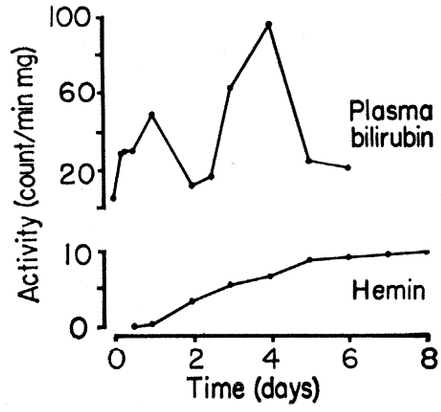

Fig. 1. Specific activity of bilirubin isolated from plasma and of hemin from circulating erythrocytes in man given 50 μc of glycine-2-C^{14} intravenously.

In a parallel experiment 12.5 μc of δ-aminolevulinic acid-4-C^{14} (California Biochemicals) was given intravenously to a 75-kg man. The appearance of labeled bilirubin in his plasma and of labeled heme in his red cells is shown in Fig. 2. Although δ-aminolevulinic acid was a poor heme precursor, labeled bilirubin appeared within 30 minutes, reached its peak at 90 minutes, and then rapidly decreased. There was no second peak. The same pattern was observed in two other subjects.

The first component of labeled bilirubin found after administration of glycine is comparable to, although slower to appear than, that found when δ-aminolevulinic acid is used as the precursor. Glycine as a precursor of

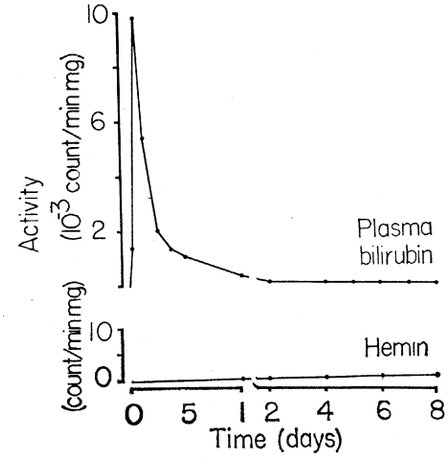

Fig. 2. Specific activity of bilirubin isolated from plasma and of hemin from circulating erythrocytes in man given 12.5 μc of δ-aminolevulinic acid-4-C^{14} intravenously.