specific for hydrolysis of the dipeptide glycyl-glycine.

*Discussion.* Any attempt at quantitating the inhibition of a metal ion on an enzymatic system in which the bulk of the protein is probably not the enzyme, as in our case, has to make allowance for the fact that a certain proportion of the metal added will be non-specifically bound to inert protein, and only a portion will be free to react with the enzyme or the substrate. This non-specific binding leads to greatest errors in low metal concentration and is of less importance at higher concentration. The curves depicting the inhibitory effect of metals on enzymatic hydrolysis of glycyl-L-leucine and triglycine (Figs. 1 and 2) are therefore open to question as to whether inhibition actually does occur with much lower concentrations of each of the metal ions studied. This cannot be answered until each of the cerebral peptidases can be tested in pure form. The extremely low concentrations of cobalt ions needed to activate diglycinase activity would tend to argue against any significant portion of this metal being non-specifically bound by inert proteins. The data do show that there is a relative difference in the inhibitory effects of the different metals, some like $Hg^{++}$ and $Cd^{++}$ being more potent inhibitors by several orders of magnitude. The effect of cobalt ions on the hydrolysis of the diglycine residue of triglycine after the N-terminal glycine is removed by a separate enzymatic system strongly suggests that the cerebral enzyme responsible for diglycinase action is very similar to that described by Smith(4) in other tissues, and as such represents a distinct and separate enzyme in the mixture of enzymes operating in our cerebral peptidase system. The very low but measurable diglycinase activity without addition of cobalt observed here (Fig. 3) and in earlier studies (2) probably reflects the activity resulting from the pre-existing tissue cobalt. It is anticipated that extension of these studies to other peptidase systems may provide clues to the nature of the "toxic" effects of some of the metal ions studied here in disase processes like Wilson's disease, lead and mrcury poisoning(5,6).

*Summary.* The effect of $Ca^{++}$, $Mg^{++}$, $Cu^{++}$, $Zn^{++}$, $Cd^{++}$, $Co^{++}$, $Mn^{++}$, $MoO_4^{=}$, $Ni^{++}$, $Fe^{+++}$ and $Al^{+++}$ was studied on the enzymatic hydrolysis, by cerebral peptidases, of glycyl-L-leucine and glycyl-diglycine. The inhibition was shown to be of different orders of magnitude for different metals. Only cobalt ions were shown to enhance the hydrolysis of glycyl-diglycine by promoting the hydrolysis of the diglycine residue in the tripeptide which normally resists hydrolysis. This effect was specific for diglycine and not applicable to the other N-glycyl dipeptides which are normally not hydrolyzed by brain.

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## Lack of Relationship Between Sialic Acid Content, Toxicity, and Lethality of *Escherichia coli.* (26837)

M. FORBES AND N. A. KUCK (Introduced by E. H. Dearborn)
*Experimental Therapeutics Research, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.*

Some strains of *E. coli* are more lethal than others for mice. The reason for this difference is not known. The possibility that sialic acid which occurs in cells of some strains may be involved in their lethality was suggested by the following considerations:

Kauffmann(1) found that the presence of certain L antigens, which occur as capsules or envelopes, was associated with increased virulence. Recently, Barry(2), in a survey of various strains of antigenically different *E. coli,* found sialic acid in certain strains with L type antigens, and not in strains with A or B type antigens. The question thus was raised: could sialic acid be related to those L type antigens whose presence had been associated with increased virulence?

We have tested the hypothesis, mentioned previously by others(3), that sialic acid in *E. coli* may play a role in enhancing their virulence or toxicity. Virulence was measured as lethality of viable cells for mice and toxicity was measured by the lethal effects of killed cells. We have further determined sialic acid content and virulence of young and older cells of the same strain and compared the toxicity of extracts from such cells.

*Method.* The study was carried out with 13 strains, each of a different serological type.* The cultures were maintained on nutrient agar slants and stored at 4°C.

The general procedure was as follows: 200 ml batches of trypticase soy broth (Baltimore Biological Labs.), inoculated with 2 ml of an overnight broth culture, were incubated for 5 hours at 37°C at which time the culture was in the upper half of its logarithmic phase. In some cases, cultures were incubated for 24 hours. An aliquot was removed and used for lethality titrations, and the remainder was centrifuged. The cells were washed once with distilled water, suspended in water and lyophilized.

For lethality titrations one-half ml portions of serial dilutions in broth of the cultures grown for 5 or 24 hour periods were injected intraperitoneally in male CF1 (Carworth Farms) mice weighing 20 $\pm$ 2 g each. The number of organisms injected was determined by plate count. Ten mice were used per infecting dose and mortality noted over a period of 7 days. Titrations were performed 3 or 4 times with each strain. $LD_{50}$ values of the individual tests, expressed as number of viable cells, were determined using an electronic computer programmed following procedures given by Finney(4). The computed values were combined to give a pooled $LD_{50}$ for each strain.†

For toxicity titrations a weighed amount of lyophilized cells was suspended in saline and killed by exposure to 100°C for 10 minutes. One-half ml of dilutions of these suspensions was injected intraperitoneally in mice. Ten mice were used per dose and mortality was observed over a period of 7 days. Toxicity titrations were performed 2 or 3 times for each strain. $LD_{50}$ values of the individual tests, expressed as mg of heat killed cells, were determined and combined to give a pooled $LD_{50}$ for each strain.

Due to interference by chromogenic materials, it was not possible to apply colorimetric tests for sialic acid directly on the lyophilized cells. Sialic acid determinations were, therefore, carried out on hydrolysates of lyophilized cells according to the procedure of Svennerholm(5). Preliminary tests showed that no loss of sialic acid occurred in washing and lyophilizing of cells. Hydrolysates were prepared by suspending lyophilized cells (3 to 15 mg/ml) in 0.1 N $H_2SO_4$ and maintaining the suspensions at 80°C for one hour after which cellular material was removed by centrifugation. Preliminary tests had shown, in agreement with the findings of DeWitt and Rowe(3), that this hydrolysis procedure was optimal. Hydrolysates of 10 to 100 mg of cellular material were passed over a Dowex 2-X-8 resin (acetate form) column. After washing the column with water the adsorbed sialic acid was eluted with 8 ml of 1 M sodium acetate-acetic acid buffer (pH 4.6), followed by a 6 ml aliquot of buffer. The column eluates were tested for sialic acid by the resorcinol method(5) using N-acetylneuraminic acid as standard, and results were expressed in terms of this compound. Deter-

* Strains of *E. coli* were kindly provided by Dr. W. H. Ewing (Communicable Disease Center, Chamblee, Ga.), Dr. C. W. DeWitt (Upjohn Co.,), Dr. G. T. Barry (Univ. of Tennessee), and Dr. M. K. Nadel (Lederle Laboratories). We wish to thank Dr. W. H. Ewing, for verifying the serological type of several strains.

† Statistical computations were carried out by Dr. W. Kwolek (Statistical Dept., Lederle Laboratories).

TABLE I. Lethality, Toxicity and Sialic Acid Content of *E. coli* of Various Serotypes.

| Strain and origin | $LD_{50}$ for mice, No. viable organisms/mouse $\times 10^5$ | Relative virulence* | $LD_{50}$ of heat killed cells, mg/mouse | Sialic acid, $\mu$g/mg cells, avg and range† |
|---|---|---|---|---|
| $O_{16}K-$ (Ewing) | 1100 (840-1400)‡ | 1 | 2.7 (2.2-3.3)‡ | <.3 |
| $O_{111}B_4$ (Nadel) | 800 (220-1600) | 1 | 2.4 (1.9-2.9) | <.3 |
| $O_{127}B_8$ ( " ) | 790 (620-1000) | 1 | 1.5 (1.0-2.6) | <.3 |
| $O_{55}B_5$ ( " ) | 370 (260- 530) | 3 | 1.6 (1.0-2.7) | <.3 |
| $O_{16}K_1$ (Ewing) | 360 (130-1000) | 3 | 3.9 (3.4-4.5) | 11 (8-17) |
| $O_9K_{29}$ ( " ) | 330 (150- 730) | 3 | 2.1 (1.0-2.5) | <.3 |
| $O_9K_{35}$ ( " ) | 180 (140- 240) | 6 | 1.2 ( .8-1.5) | <.3 |
| $O_{26}B_6$ (Nadel) | 92 ( 60- 140) | 12 | 1.5 (1.2-1.8) | <.3 |
| $O_{10}K_7$ (Barry) | 15 ( 12- 20) | 70 | 1.5 (1.2-1.9) | <.3§ |
| $O_1K_1$ (Ewing) | 13 ( 8- 20) | 90 | 1.3 (1.1-1.6) | 15 (9-17) |
| $O_8K_{44}$ ( " ) | 6 ( 5- 10) | 180 | 2.0 (1.6-2.4) | <.3 |
| $O_7K_1$ ( " ) | 3 ( 1- 4) | 400 | 1.9 (1.6-2.3) | 10 ( 6-13) |
| $O_2K_1$ ( " ) | 0.4( 0.1- 3) | 2700 | 1.7 (1.3-2.1) | 14 (10-17) |

* Relative to virulence of strain of serotype $O_{16}K-$.

† Avg and range of determinations on 2 to 5 batches of cells as determined by the Svennerholm procedure using N-acetylneuraminic acid as standard.

‡ Figures in parentheses are 95% confidence limits.

§ Small amounts of sialic acid have been reported for this strain(2).

minations on duplicate and triplicate hydrolysates of the same batch of cells gave sialic acid values which checked within 20% of each other. Samples were also checked by the Ehrlich(6) and the thiobarbituric acid (7) methods. Values for sialic acid found by using the Ehrlich test agreed within experimental error with the results of the resorcinol test. The thiobarbituric acid test was complicated, however, by the presence in some *E. coli* strains of material which gave no reaction in the resorcinol and Ehrlich tests, but which with thiobarbituric acid gave a chromophore with an absorption peak close to that produced by sialic acid.

The efficacy of the system was tested by recovery studies. Sialic acid (30 to 100 $\mu$g) was added to 10 to 100 mg batches of cells prior to hydrolysis. Recovery of added sialic acid was 90 to 100%. The lower limit of detection was 0.3 $\mu$g/mg cells. Thirty $\mu$g of sialic acid added to 100 mg of cells could readily be detected.

The presence of sialic acid in the bacteria was confirmed by paper chromatography. Concentrates of the column eluates from which the sodium was removed by passing them over a Dowex 50-X-8 (H+ form) resin column were chromatogrammed in 3 systems(8): 1-butanol:acetic acid:water (4:1:5); 2-butanol:acetic acid:water (4:1:5) and ethyl acetate:pyridine:acetic acid:water (5:5:1:3). The chromatograms were developed with a resorcinol spray(9). Samples‡ of N-acetylneuraminic acid and N-acetyl-4-O-acetylneuraminic acid served as standards and were run in parallel or mixed with the concentrates of the column eluates.

*E. coli* were disintegrated by shaking suspensions of cells (25 mg/ml) in water with powdered glass in a vial clamped on a shaker head (International Equipment Co. No. 6007) run at 1400 rpm for 20 minutes in an International centrifuge. The supernatant of the shaken suspension was filtered through (U.F.) sintered glass filters. The ability of these cell-free lysates to cause hemorrhagic lesions was tested by injecting aliquots combined with epinephrine (100 $\mu$g) in total volumes of 0.2 ml intradermally in the shaved abdomen of rabbits according to the procedure described by Thomas(10).

*Results.* In Table I the *E. coli* strains are listed in order of increasing lethality as determined by their $LD_{50}$ values. For convenience, the strain of serotype $O_{16}K-$, which was the least lethal, was used as standard and the lethality of the other strains was expressed in relation to this strain. Using this

‡ A sample of N-acetylneuraminic acid was kindly provided by Dr. R. J. Winzler (Univ. of Illinois) and samples of diacetylneuraminic acid were kindly donated by Dr. Gunnar Blix (Uppsala Univ., Sweden).

TABLE II. Lethality and Sialic Acid Content of *E. coli* Strains Grown for 5 and 24 Hour Periods.

| Serotype of strain and origin | Age of culture, hr | $LD_{50}$ for mice, No. viable organisms/mouse | Sialic acid, μg/mg cells, avg and range |
|---|---|---|---|
| $O_2K_1$ (DeWitt) | 5 | .3 ( .1- .6) × $10^5$ | 14 (10-17) |
| | 24 | .5 ( .3- 1.0) × $10^5$ | <.3 |
| $O_1K_1$ (Ewing) | 5 | 37 ( 7 -57 ) × $10^5$ | 15 ( 9-17) |
| | 24 | 56 (37 -87 ) × $10^5$ | <.3 |
| $O_7K_1$ ( " ) | 5 | 7 ( 4 - 9 ) × $10^5$ | 10 ( 6-13) |
| | 24 | 13 ( 9 -18 ) × $10^5$ | <.3 |

scale, for instance, the strain of serotype $O_{111}B_4$ is as lethal as the $O_{16}K$- strain, but the strains of serotype $O_{26}B_6$, $O_1K_1$ and $O_2K_1$ respectively are 12, 90 and 2700 fold more lethal.

The difference in lethality between strains is not accounted for by differences in heat stable toxins in the cells. $LD_{50}$ values of the heat killed cells of the various strains did not differ significantly, with the possible exception of the strain of serotype $O_{16}K_1$ which appeared to be slightly less toxic than the other strains. (Table I).

The difference in lethality between strains is not accounted for by differences in sialic acid content. Three of the 5 most lethal strains, those of serotype $O_2K_1$, $O_7K_1$ and $O_1K_1$ were found to contain from 1 to 1.5% sialic acid (Table I). No sialic acid was found in the strain of serotype $O_8K_{44}$. Similarly no sialic acid was detected in hydrolysates of 200 to 300 mg of cells of the strain of serotype $O_{10}K_5$ although this strain has been reported(4) to contain small amounts of the acid. On the other hand the strain of $O_{16}K_1$ which is relatively avirulent contained as much sialic acid as each of the 3 more lethal strains. Paper chromatograms of hydrolysates of all 4 strains with the $K_1$ antigen showed the presence of resorcinol positive material which in 3 different systems could not be separated from a sample of authentic N-acetylneuraminic acid. No spots corresponding to N,O-diacetylneuraminic acid were observed; this substance, however, is known to be somewhat unstable and may degrade to the N-acetyl derivative(3). Our finding of sialic acid in strains containing the $K_1$ antigen is in agreement with observations by Barry(2).

During further studies we noted that the sialic acid content of cells grown in trypticase soy broth gradually decreased as the cells became older, to the point that in cells from cultures from 18 to 24 hours old none could be detected. This loss of sialic acid in the older cells was not permanent, for when these cells were used as inoculum in fresh broth, they proliferated and produced offspring containing sialic acid. The observation that older cells in contrast to younger cells contained little, if any, sialic acid offered the opportunity to compare the effect on lethality of the presence or absence of sialic acid in cells of the same strain. Differences in survival of the inocula in the host would be expected to be reflected in differences in lethality. Comparison of the lethality of 5 and 24 hour old cultures of strains of serotype $O_2K_1$, $O_7K_1$ and $O_1K_1$ are shown in Table II. Titrations yielding the $LD_{50}$ values shown in Table II were performed about 12 months after the data shown in Table I were obtained. It can be seen by comparing the $LD_{50}$ values for the 5 hour cultures of the 3 strains both in Table I and Table II that their lethality remained relatively constant. The 24 hour old cells of all 3 strains are only slightly less lethal than the corresponding 5 hour old cells (Table II); yet they contain, if any, only a fraction of the sialic acid present in the 5 hour old cells. No sialic acid was detected in hydrolysates made by exposing 100 mg of cells from 24 hour cultures to 0.1 N or 1 N $H_2SO_4$ at 80°C for one hour. In contrast, sialic acid was readily detected and measured in hydrolysates of as little as 15 mg of 5 hour cells of the strains of serotype $O_1K_1$, $O_2K_1$ and $O_7K_1$.

Cell-free lysates of 5 hour cells of the

strain of serotype $O_2K_1$ contained sialic acid in bound form. This sialic acid could be released by mild hydrolysis, as verified by the thiobarbituric acid test which measures only free sialic acid, and as confirmed by paper chromatography. Aliquots of such lysates, containing 30 to 50 $\mu$g of N-acetylneuraminic acid in bound form, injected intraperitoneally in mice caused toxic symptoms including anorexia, loss of weight, lethargy and occasionally death. However, comparable lysates of 24 hour old cells of the same strain, which contained no sialic acid, caused the same symptoms when injected in mice. Further when injected intradermally in combination with epinephrine in the shaved abdomen of rabbits, both cell-free preparations produced hemorrhagic lesions at sites of the injections. These results indicated that sialic acid played no essential role in the toxicity of these cell extracts.

*Discussion.* The data tabulated in Table I show that there is no correlation between sialic acid content of cells of strains of *E. coli* and lethality for mice. Increased lethality must depend on survival of a small inoculum in the host and it is during the lag phase that the inoculum would be expected to be most vulnerable to host defense mechanisms. The 24 hour old cells which contained little, if any, sialic acid were as lethal and hence as able to survive in the host as the 5 hour cells which contained as much as 1 to 1.5% sialic acid (Table II). Hence, sialic acid does not play an essential role in protecting the cells during their lag phase from the host defense mechanism.

*Summary.* The lethality of 13 *E. coli* strains, each of a different serological type, was compared in mice and was found to differ greatly. These differences could not be attributed to differences in heat stable toxins nor to presence or absence of sialic acid in the cells. *E. coli* strains containing sialic acid gradually lost most, if not all, of this monosaccharide after 24 hours growth in culture, but no corresponding decrease in lethality occurred. Cell-free lysates of *E. coli* containing bound sialic acid were toxic for mice, and when injected in combination with epinephrine produced hemorrhagic lesions in rabbits, but no more so than did comparable lysates from older cells of the same strain which contained no sialic acid.

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## Dietary Composition and Tissue Protein Synthesis I. Effect of Tryptophan Deficiency.* (26838)

MARCELO E. NIMNI AND LUCIEN A. BAVETTA
*Department of Biochemistry and Nutrition, School of Dentistry, University of Southern California, Los Angeles*

Protein deficiency is a well known factor in growth retardation. A low dietary level of an adequate protein or of one of the essential amino acids in an otherwise complete diet is the most common way of inducing a protein deficiency. Protein free diets or com-

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