STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

X. Pimelic Acid as a Growth Stimulant for C. diphtheriae

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In the preceding paper (Mueller and Subbarow, 1937) it was shown that the constituents of a tissue extract (liver) essential for heavy growth of a strain of *Corynebacterium diphtheriae* were at least two in number, and that they could be separated from each other by ether extraction of an acidified concentrate. Since the amount of total material passing into the ether was only a small fraction of that remaining in the aqueous phase, and further, because the solubilities of the former gave a definite clew as to its probable chemical nature (organic acid or phenol), it seemed wise to attempt the isolation of this substance first, leaving a further study of the other, perhaps still multiple, factor until later.

It is clear that in studies of this sort, involving an unknown number of variables, it is difficult, and yet essential so to plan the work that a control solution shall be available, and susceptible to ready duplication, which will give an unmistakable indication of the presence or absence of a fraction under investigation over a considerable period of time. After various preliminary experiments which indicated clearly that the phenomenon to which reference is made could be produced regularly, and that the ether-soluble material was probably an organic acid and not a phenol, a control solution which seemed likely to be adequate to the needs of the work was arranged. The basis for this was the formula previously used, a casein hydrolysate,—enriched by cystine and glutamic acid both of which are present in less than optimal con-

centration when one per cent of casein is employed,—together with suitable inorganic salts and sodium lactate. To this had to be added the ether insoluble fraction of the liver concentrate. A whole protein hydrolysate is used, rather than pure amino acids, in spite of the certain presence of unknown substances, for two reasons. First, should it be essential for any reason to employ a different test strain in the course of the work, a considerable delay might be anticipated in arriving at a suitable amino-acid formula, because of the considerable differences between various cultures of the diphtheria bacillus. In the second place, the expense involved is negligible as compared with that connected with the extended use of pure amino acids.

A uniform supply of casein hydrolysate, adequate for a long series of experiments, was assured by hydrolyzing several pounds of casein with concentrated HCl, evaporating to a thick gum, then dissolving in water to a concentration corresponding to 20 per cent of the protein and treating with norit charcoal at room temperature until only a pale straw color remained after filtration. Toluol was added and the material kept in the cold room, although such a preparation will keep for long periods at room temperature without preservative because of the highly acid reaction.

The ether-insoluble liver fraction was prepared from the calcium-alcohol filtrate described in the last paper (IX), using a quantity corresponding to 60 kgm. of liver tissue. The details of the preparation need not be given. Essentially it consisted in butyl-alcohol extraction of the acidified solution, the transfer of the extracted material to water followed by removal of the ether-soluble fraction. The remaining material, thoroughly dried, was extracted with absolute methyl alcohol, and the solution precipitated fractionally with ether. The most active fraction (the third) was dissolved in 50 cc. hot absolute ethyl alcohol and after cooling over night in the ice box was filtered from a small amount of separated solid. Of this solution 0.01 cc. in 10 cc. of medium equivalent to 12 grams of fresh liver tissue, proved to be adequate for heavy growth in the presence of the other constituents of the

control solution plus a sufficient amount of the ether-soluble fraction. The solution contained 0.0428 gram material per cubic centimeter. The quantity was sufficient for 5000 test lots of media. At the conclusion of the experiments about half of the solution remains. Reference is made to this solution in the protocols as "4b."

The course of the experiments may be traced briefly, after which representative experiments will be presented to illustrate the more important steps.

Assuming the active material to be one or more organic acids, esterification, followed by vacuum distillation was first carried out, and found to yield a distillate active after hydrolysis. Through the courtesy of Pappenheimer (1935), we were able to test some of his "sporogenes vitamin" having similar properties but obtained no particular effect from its use. Having in mind, however, his finding of the substance in urine, as well as in tissue and yeast extracts, we examined the urine of several different species of animals for the diphtheria ether-soluble fraction. The experiments indicated the presence of material which adequately replaced the liver acid. Particularly in the urine of the horse and cow, it evidently occurred in greater concentration than in fresh liver tissue. This active material, too, proved to be ether-soluble (from acid solution), and to be esterifiable and to distill in vacuum presumably as the ester. Through the courtesy of several commercial houses which manufacture theelin from urine, and of various experimental workers in the field, certain concentrates containing the organic acids of urine were tested, but none were found suitable for use. Suspecting wide differences in partition of the substance between water and various organic solvents, the point was tested, and butyl alcohol, ethyl acetate and ethyl ether proved able to extract the material easily, while benzene, chloroform and petroleum ether failed to remove appreciable amounts.

Preliminary experiments on concentrates prepared from small quantities of human and horse urine indicated that the distilled esters were a mixture of several substances, yielding on hydrolysis

a discouraging oily material, and that the greatest hope of success lay in working through sufficient material to carry out adequate fractional distillations.

One hundred gallons of cows' urine, calculated to yield between 50 and 100 grams of distilled methyl ester were therefore processed. A detailed account of the method from the chemical point of view will be presented elsewhere. It included preliminary extraction of the acidified urine with butyl alcohol, instead of ether, removal from the solvent with $NaHCO_3$ solution, separation of a large amount of hippuric acid by acidification and crystallization, and then passing back and forth between butyl alcohol and aqueous sodium bicarbonate, gradually reducing the volume and at each transfer eliminating considerable quantities of inert material. A barium-hydroxide alcohol precipitation greatly aided the purification, the active substance appearing in the filtrate from the bulky precipitate. Esterification was finally effected in absolute methyl alcohol with HCl, a further purification at this stage resulting from the removal of non-ether-soluble esters (phenaceturic acid, etc.) and non-esterifiable material by extraction with $NaHCO_3$ and finally weak NaOH.

The resulting esters, after drying, yielded 83 grams vacuum distillate up to 163°C., using an oil pump vacuum of less than 1 mm. It was separated empirically into five fractions by means of a Fischer triangle as it passed over. Eighty grams of dark brown residue remained in the flask at the end of the distillation. All the fractions showed activity, but this was most pronounced in the lower-boiling portions. Various types of redistillation were tried on small lots, particularly on fractions resulting from earlier small scale experiments. It was found that the material was surprisingly stable, and would withstand even atmospheric pressure distillation, passing over with no evidence of decomposition and fully active at about 250°, but still too impure to deal with the regenerated acids.

At this point we learned of a small specially constructed vacuum distilling column designed and being used by Dr. David Rittenberg of the Department of Biochemistry at the College of

Physicians and Surgeons in New York, with which he was able to separate almost quantitatively small amounts of substances with boiling points only a few degrees apart. We were fortunate in being able to enlist Dr. Rittenberg's cooperation and not only learned from him how to construct and operate his column, but actually watched him run a distillation on one of our active fractions. From about three grams of our esters he separated ten fractions, and subsequent test showed a very satisfactory degree of concentration in the middle boiling portions.

After setting up an apparatus similar to his, the most active of the total ester fractions were systematically distilled, and the most active of the resulting fractions re-distilled. Tests indicated the highest activity in two adjoining fractions in about the middle of the range covered. The lower boiling of these remained a clear, thick syrup even when cooled in carbonic ice and alcohol, whereas the higher one set to a solid mass of fine crystals. It was decided to hydrolyze this latter fraction, amounting to 2.5 cc. About half of it was boiled with alcoholic NaOH, acidified, evaporated to dryness and extracted with ether. Removal of the ether left a definitely granular material mixed with some oil. In this impure form difficulty was next experienced in finding a suitable solvent with which to attempt recrystallization. The substance proved to be practically insoluble in some, and much too readily soluble in other solvents. Dibutyl ether was finally employed and from this, crystallization readily took place, the material being practically insoluble at room temperature, but dissolving easily on heating. After three recrystallizations the melting point appeared to be constant at 104° to 105°, and granular white crystals, 1 to 2 mm. in diameter were formed. The yield was 0.3 gram.

The crystals proved to be highly active in growth tests. Titration with N/100 NaOH, semi-micro combustions, micro-molecular weight determinations and the preparation of the phenylphenacyl ester of the acid and a molecular weight determination on this gave results consistent with the formula $C_5H_{10}(COOH)_2$. The dibasic acid of this formula, pimelic acid, is stated to melt at

105°, as did our crystals. Mixed melting points with Eastman's pimelic acid showed no lowering, and growth tests with the latter were in every way identical with those carried out on our isolated crystals. The effect becomes apparent at a concentration of about 0.005γ of pimelic acid and reaches a maximum at about 0.025γ, per cubic centimeter of culture medium. An excess of the substance produces no further change even up to a concentration of 1 per cent.

The next lower boiling fraction of esters, showing almost as great an activity as the one used above was worked up in the same way, and from this, also, pimelic acid was readily obtained. More impurity was present, and it was necessary to recrystallize repeatedly from butyl ether, and finally twice from water—with considerable loss—before good crystals with the correct melting point were obtained. The other material in this fraction was not further investigated.

From an earlier preparation of esters on a smaller scale there had been obtained a small amount of a crystalline hydrazide, from which it was possible to prepare and recrystallize a substance which proved to be azelaic acid, $C_7H_{14}(COOH)_2$. A growth test with this material proved negative, and since the sticky, non-crystalline impurities were highly active, azelaic acid was not investigated further at that time. After the isolation of pimelic acid, however, all the dibasic acids up to and including azelaic were carefully tested. None of these were found to have an effect in any way comparable with that of pimelic acid.

It should be added that a certain amount of growth acceleration was produced by another of the ester fractions, one of the lower boiling ones. This material is definitely different from the esters of the dibasic acid. It has a highly aromatic odor, somewhat suggestive of that of methyl benzoate, and has given crystals which are probably phenyl acetic acid. The effect of these crystals, as of phenyl acetic acid itself, is irregular, heavy growth not being produced in any case, and adding them to pimelic acid gives no better growth than the pimelic acid alone.

There is no indication at present as to the mechanism for this peculiar action of a compound which, as far as the writer can

learn, has not previously been encountered in biological material. Its action is not limited to our test strain (Allen), but is manifested with at least two strains of Park 8, one from the National Institute of Health, the other from the Alabama State Department of Health. Whether it will prove to stimulate the growth of bacteria other than the diphtheria bacillus has not been investigated. Suggestive, however, are the findings of Snell, Tatum and Peterson (1937) and Wood, Tatum and Peterson (1937) that unidentified organic acids are necessary for growth of certain lactic and propionic bacteria. Professor Peterson has kindly tested for the writer a still impure, but active preparation containing pimelic acid, on his lactic strain and found it to be without effect. Dr. Tatum, now in Utrecht, writes that the acids concerned in growth of his propionic organisms are extracted with ether only with the greatest difficulty. Probably further work will indicate whether these higher dibasic acids have a more general biological significance.

It remains to express again the writer's sincere appreciation of the cooperation given so freely by Dr. Rittenberg. Without his assistance the completion of this problem would have been infinitely more difficult.

EXPERIMENTAL

The control solution for 10 cc. of medium consisted of the following materials, and unless otherwise noted, remains constant in subsequent experiments:

Casein HCl hydrohysate	0.100	gram
Cystine	0.001	gram
Glutamic acid hydrochloride	0.050	gram
NaCl	0.050	gram
$Na_2HPO_4 \cdot 2H_2O$	0.025	gram
KH_2PO_4	0.0035	gram
$MgCl_2 \cdot 6H_2O$	0.003	gram
Lactic acid (as Na salt)	0.10	cc.

The "Allen" strain* (paper IX) was used as test organism, and the amount of growth is given in terms of milligrams of bacterial nitrogen after 48 hours' growth.

I. The ether-soluble factor from liver extract may be esterified and distilled

Six hundred cubic centimeters of the same calcium-alcohol-ether filtrate of a liver concentrate used in the experiment of March 6, 1936, and described in the previous paper, were employed. This was equivalent to approximately 60 kgm. of fresh liver tissue. It was diluted to 2400 cc., stirred for five minutes with 75 grams Fuller's earth and filtered, and then treated similarly with 30 grams norit charcoal, and finally with 10 grams more norit. After the third filtration the solution was only slightly yellowish in color. It was concentrated in vacuo to 175 cc.

After making alkaline by the addition of 17.5 cc. of 10 N NaOH it was extracted five times with 200 cc. butyl alcohol, then neutralized with concentrated HCl, and 17.5 cc. excess added. Ten extractions with 200 cc. of peroxide-free ether were then carried out, followed by five with 200 cc. butyl alcohol.

The combined wet ether extracts were evaporated to about 30 cc. in vacuo. The residue, largely water, was transferred to a 500-cc. boiling flask; frozen around the wall in dry ice and transferred to the Flosdorf-Mudd drying apparatus, and the water thoroughly removed by oil pump vacuum for 18 hours. The residue consisted of 1.3 grams of a mixture of crystals and a thick yellow oil. The latter was largely removed by successive washings with small amounts of ether, and was shown to contain the active substance. The crystals (succinic acid?) were without activity, and weighed about 0.15 gram.

The ether was removed from the solution of the oily material, which was then dissolved in 10 cc. absolute methyl alcohol. To this was added 1.0 cc. of a 30 per cent solution of dry HCl in methyl alcohol. The mixture was heated briefly to boiling, and allowed to cool slowly and stand without further heating for two hours. As much alcohol and HCl as possible were then removed by vacuum distillation, and the residue dissolved in ether and washed successively with saturated $NaHCO_3$, 0.5 per cent NaOH, and water. The ether solution of the esters was then dried with Na_2SO_4, transferred in small portions to a Wassermann tube and the ether removed in a current of warm dry air. The residue, presumably methyl esters, weighed 0.225 gram.

After some preliminary experiments using a few milligrams of the material, the remainder, of perhaps 0.2 gram of esters in the small test tube was subjected to vacuum distillation. The test tube was sealed to a long narrow glass tube which was then bent to form a narrow U-shaped trap. The esters were heated in an oil bath to 120° for about an hour, the trap being cooled with dry ice, and a vacuum of less than 1 mm. applied from an oil pump. Distillate collected both in the trap and in the air-cooled section of the apparatus. No attempt was made to secure different fractions. After releasing the vacuum and bringing the apparatus to room temperature, the portion of trap and connecting arm containing the distillate was cut away from the rest of the apparatus. About one-third of the material appeared to have distilled. It was a water-clear liquid with a very faint odor somewhat resembling that of malonic ester.

About 0.0002 gram of the ester was transferred to 10 cc. N/1 NaOH and heated fifteen minutes in the water bath to effect saponification, and growth tests prepared as follows:

	mgm. N
1. Control + HCl-butyl alcohol extract	1.06
2. Control + HCl-butyl alcohol extract + saponified esters 0.1 cc.	1.62
3. Control + HCl-butyl alcohol extract + saponified esters 1.0 cc.	2.86

II. The ether-soluble factor is not identical with "Sporogenes vitamin"

Through the kindness of Dr. Pappenheimer a small quantity of the distilled ester of this material was made available for testing. Two milligrams were saponified by autoclaving for ten minutes at 10 pounds pressure with 2 cc. N/1 NaOH, the following test carried out:

	mgm. N
1. Control + 4b[1] + 0.1 mgm. sporogenes vitamin ester	1.90
2. Control + 4b + 0.01 mgm. sporogenes vitamin ester	0.66
3. Control + 4b + 0.001 mgm. sporogenes vitamin ester	0.66
4. Control + 4b + 0.0001 mgm. sporogenes vitamin ester	0.74
5. Control + 4b + 0.00001 mgm. sporogenes vitamin ester	0.68
6. Control + 4b	0.64

[1] See page 165.

Since the sporogenes vitamin manifests activity in quantities of small fractions of a gamma, and since from *I* it is evident that the liver acid is active in quantities of less than 0.01 mgm. even in an impure form, it is clear that the two are not identical. The growth with 0.1 mgm. may have been caused by a trace of impurity in the sporogenes preparation or, the substance itself may have an effect of its own at this concentration.

III. The ether-soluble fraction of liver extract can be replaced by urine

	mgm. N
1. Control + 4b +	0.70
2. Control + 4b + human urine 1.0 cc	3.44
3. Control + 4b + human urine 0.1 cc	3.56
4. Control + 4b + human urine 0.01 cc	1.30
5. Control + 4b + mare's urine 0.1 cc	3.16
6. Control + 4b + mare's urine 0.01 cc	2.16
7. Control + 4b + mare's urine 0.001 cc	0.70
8. Control + 4b + cow's urine 0.1 cc	3.24
9. Control + 4b + cow's urine 0.01 cc	3.26
10. Control + 4b + cow's urine 0.001 cc	1.04

IV. Extraction of active substance from urine by various solvents

Cow's urine was acidified by adding 25 cc. concentrated H_2SO_4 per liter, and after standing over night, a small amount of kieselguhr was added, and the material filtered by gravity through a pleated filter paper. Twenty-five-cubic centimeters portions of the filtrate were thoroughly shaken with 25 cc. quantities of the following solvents, with results as shown:

1. Butyl alcohol	Considerable material extracted
2. Ether	Considerable material extracted
3. Ethyl acetate	Considerable material extracted
4. Chloroform	Small amount of material extracted
5. Benzene	Small amount of material extracted
6. Petroleum ether	Practically nothing extracted

After evaporation of the solvent, the extracted materials were taken up in 25 cc. water each, with a little NaOH to effect solution.

Growth tests showed the first three extracts to be highly active, the others completely inactive.

V. The preparation and distillation of esters from 100 gallons of cow's urine

This was carried out in daily lots of 10 gallons, acidifying with H_2SO_4, filtering, and extracting three times with about one-third volume of butyl alcohol, using 5-gallon bottles for the procedure. The butyl alcohol was in turn extracted with a suspension of $NaHCO_3$ in water, to alkaline reaction, with thorough and prolonged mixing by means of an air current, and the aqueous solution of sodium salts of the acids combined from day to day, until the entire amount had gone through this stage. In this way there was obtained about 10 gallons of concentrate. The further purification of this material will be described in detail in another place. Eventually, there was obtained 120 grams of dry methyl esters ready for distillation, and possessing a satisfactory degree of activity in growth tests.

Distillation was carried out from a 500-cc. Claissen flask, using a Fischer triangle, dry ice trap, and oil pump vacuum of less than 1 mm. Hg. The following fractions were taken:

	TEMPERATURE OF VAPORS	TEMPERATURE OF OIL BATH	WEIGHT OF FRACTION
	degrees	*degrees*	*grams*
I	81–84	85–114	15
II	93–109	135	19
III	110–130	155	25
IV	130–149	175	11
V	158–163	195	13

A few milligrams of each of these distillates, as well as of the residue, were weighed out and saponified 1 mgm per cubic centimeter with N/1 NaOH. An abbreviated portion of the test with these solutions shows the following comparative effects on growth:

	mgm. N
1. Control + 4b + distillate I, 1.0γ	1.46
2. Control + 4b + distillate II, 1.0γ	1.58
3. Control + 4b + distillate III, 1.0γ	1.14
4. Control + 4b + distillate IV, 1.0γ	0.98
5. Control + 4b + distillate V, 1.0γ	0.88
6. Control + 4b + residue, 1.0γ (nitrogen not done, no increase).	
7. Control + 4b	0.70

Fractions I and II were re-distilled separately under atmospheric pressure, mixing the distillates of the second with those of the first having corresponding boiling points. Distillation was carried out slowly, from an oil bath. The following fractions were obtained:

	VAPOR TEMPERATURE	WEIGHT OF DISTILLATE	
		From fracture I	From fracture II
	degrees	*grams*	*grams*
A	198–210	2.5	
B	210–220	5.9	
C	220–225	2.2	
D	228	0.8	
E	233–245	1.4	3.0
F	245–255	0.5	5.0
G	255–265		6.3
H	265+ (both to 300°)		4.5

Residue 3 to 4 grams.

Growth tests made on these fractions, after hydrolysis, as before, showed little activity in fractions A and B, and in the residue. Values for the others resulted thus;

	mgm. N
1. Control and 4b + fraction C, 1.0γ	1.34
2. Control and 4b + fraction D, 1.0γ	1.86
3. Control and 4b + fraction E, 1.0γ	2.30
4. Control and 4b + fraction F, 1.0γ	2.24
5. Control and 4b + fraction G, 1.0γ	1.96
6. Control and 4b + fraction H, 1.0γ	1.76
7. Control and 4b	0 62

The following distillation was carried out by Dr. Rittenberg on 3.1 grams of fraction F. The description of the apparatus will be published shortly by Dr. Rittenberg in connection with his own studies. It consists essentially in a very slow vacuum distillation with the use of an effective fractionating column. Under a vacuum of about 0.04 mm. Hg the following fractions were obtained in about seven hours of actual distillation:

	TEMPERATURE OF VAPORS	APPROXIMATE QUANTITIES
	degrees	*cc.*
1	? (low)	0.7
2	? (low)	0.3
3	50–74	0.4
4	72	0.2
5	78–80	0.3
6	82	0.3
7	80	0.15
8	86	0.2
9	87	0.1
10	Residue	0.4

These distillates were entirely colorless liquids, the first four of which became thick when cooled in dry ice and alcohol but showed no evidence of crystallizing, whereas the later fractions and the residue solidify to a mass of white crystalline material, quickly melting again at room temperature. The first two fractions had a definitely aromatic odor, whereas the others were almost odorless.

Growth tests gave the following results:

	mgm. N
1. Control + 4b + fraction 1, 1.0γ	1.88
2. Control + 4b + fraction 2, 1.0γ	2.60
3. Control + 4b + fraction 3, 1.0γ	2.58
4. Control + 4b + fraction 4, 1.0γ	3.12
5. Control + 4b + fraction 5, 1.0γ	3.26
6. Control + 4b + fraction 6, 1.0γ	lost
7. Control + 4b + fraction 7, 1.0γ	2.70
8. Control + 4b + fraction 8, 1.0γ	2.48
9. Control + 4b + fraction 9, 1.0γ	2.18
10. Control + 4b + residue, 1.0γ	2.22

Growth in tube 6 appeared as good as in tubes 4 and 5, and there has consequently been a very considerable concentration of active material into fractions 4, 5 and 6.

The further separation of the bulk of the esters obtained by the atmospheric pressure distillation by means of an apparatus similar to Dr. Rittenberg's need not be given in detail. Beginning with the lowest boiling, each fraction in turn was distilled, using a vacuum of 7 mm. because of the comparatively low boiling

point, with an oil pump vacuum, and separated into several smaller ones. Those of similar boiling points and high activity resulting from different atmospheric pressure fractions were combined and again carefully fractionated. At last a fraction of about 2.5 cc. was obtained boiling between about 110° and 117° at 7 mm. pressure, which was the lowest boiling fraction to solidify when immersed in dry ice, and which corresponded in a general way with fraction 5 of Dr. Rittenberg's distillation.

The test on this fraction, after hydrolysis, follows:

	mgm. N
1. Control + 4b and active fraction, 1.0γ	2.88
2. Control + 4b and active fraction, 0.5γ	3.11
3. Control + 4b and active fraction, 0.25γ	2.82
4. Control + 4b and active fraction, 0.1γ	2.30
5. Control + 4b and active fraction, 0.05γ	2.27
6. Control + 4b and active fraction, 0.025γ	1.72
7. Control + 4b and active fraction, 0.01γ	1.08

VI. The active substance is pimelic acid

Half of this fraction yielded 0.3 gram pimelic acid. (Details of analyses, etc., to be published elsewhere.)

The following experiment, put up in duplicate, compares the effect of the natural pimelic acid, isolated from cow's urine with the Eastman synthetic product.

	A	*B*
1. Control + 4b + natural pimelic acid, 1.0γ	4.08	4.05
2. Control + 4b + natural pimelic acid, 0.5γ	3.96	4.05
3. Control + 4b + natural pimelic acid, 0.25γ	3.61	3.51
4. Control + 4b + natural pimelic acid, 0.1γ	2.48	2.28
5. Control + 4b + natural pimelic acid, 0.05γ	1.78	1.81
6. Control + 4b + natural pimelic acid, 0.025γ	1.68	1.63
7. Control + 4b + natural pimelic acid, 0.01γ	1.46	1.44
8. Control + 4b + synthetic pimelic acid, 1.0γ	4.01	3.93
9. Control + 4b + synthetic pimelic acid, 0.5γ	3.85	3.76
10. Control + 4b + synthetic pimelic acid, 0.25γ	3.58	3.54
11. Control + 4b + synthetic pimelic acid, 0.1γ	2.14	2.24
12. Control + 4b + synthetic pimelic acid, 0.05γ	1.94	1.80
13. Control + 4b + synthetic pimelic acid, 0.025γ	1.52	1.62
14. Control + 4b + synthetic pimelic acid, 0.01γ	1.49	1.24
15. Control + 4b	1.68	1.68

VII. The action of pimelic acid is not common to related dibasic acids

To determine this point, a number of experiments have been carried out similar to the one which follows:

1. Control + 4b + oxalic acid, 1.0γ..... 1.08
2. Control + 4b + malonic acid, 1.0γ..... 1.18
3. Control + 4b + succinic acid, 1.0γ..... 1.06
4. Control + 4b + glutaric acid, 1.0γ..... 1.00
5. Control + 4b + adipic acid, 1.0γ..... 1.92[2]
6. Control + 4b + pimelic acid, 1.0γ..... 3.91
7. Control + 4b + suberic acid, 1.0γ..... 1.11
8. Control + 4b + azelaic acid, 1.0γ..... 1.14
9. Control + 4b..... 1.10

VIII. The effect of pimelic acid is not limited to the Allen strain, but extends to the Park 8 strain

The following experiment was set up, using exactly the same control as for the Allen strain, except that 1 mgm. tryptophane was added to each tube. Two strains of Park 8 which differ considerably from each other in certain cultural requirements were used, one from the National Institute of Health in Washington, the other from the Alabama State Department of Health. These cultures had been recently received from the two laboratories, and kept by daily transfer on peptone infusion broth at 34° until used.

	ALABAMA STRAIN	NATIONAL INSTITUTE STRAIN
Control + pimelic acid 10.0γ.....	2.74	3.08
Control.....	1.94	1.19

CONCLUSIONS

1. The acid-ether extractable substance from liver which stimulates the growth of the diphtheria bacillus can be replaced by a substance present in urine showing similar properties.

[2] Further tests with adipic acid failed to confirm this result.

2. From the latter source it has been isolated in pure form and identified as pimelic acid, $C_5H_{10}(COOH)_2$.

3. The effect on growth of synthetic pimelic acid is identical with that of the natural substance, becoming evident at a concentration of about 0.005 gamma per cubic centimeter of medium, and reaching a maximum at approximately five times this amount. Further increase, up to 1 per cent, has no further effect and carries no inhibition.

4. Azelaic acid, $C_7H_{14}(COOH)_2$ has also been isolated from the urine preparation, but shows no effect on growth of the diphtheria bacillus, nor do any of the other simple dibasic acids from oxalic up to suberic, $C_6H_{12}(COOH)_2$.

5. So far as can be learned, pimelic acid has not previously been recognized as occurring in biological, material, and azelaic acid is not listed as a normal constituent of urine.

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