3. Aversion to the diet or improper intestinal fat absorption were not responsible for the results. 4. It was concluded that rancid fats are not toxic *per se* and that, under natural conditions, the "lard factor" may play an important part in the prevention of the disease ascribed to vit. A deficiency.

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## Progressive Decreased Glucose-tolerance and Glycogen-storage Following Acetoacetate Injection; Prevention by Insulin and Amellin. (18187)

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(Introduced by Arnold Lazarow)
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The hypothesis(1) that the gradual accumulation of intermediary fat metabolites such as beta-hydroxybutyric acid and acetoacetic acid might primarily be responsible for the progressive development of clinical diabetes and that these metabolites might exert a stimulating effect on the insulin secreting mechanism of the islet cells and ultimately lead to beta cell exhaustion through excessive strain, have been strengthened by recent publications from this laboratory(2,3, 4) and supported by Lazarow(5). He accounts for the increased fasting blood sugar levels, which are observed in men placed on a high fat diet(6,7) on the basis of this hypothesis. Houssay and Martinez(8) investigated the effect of different diets on the sensitivity of rats to alloxan and found that the toxic and diabetogenic effects were much more pronounced in the animals receiving a high fat diet. The harmful effects of a high fat diet were also observed by Opie(9), Dunham and Brunschwig(10), McKibbin *et al.*(11) and many others. It has recently been shown by Nath, Chakrabarti and Hatwalne(12) and Nath and Chakrabarti (13) that the injection of the sodium salt of beta-hydroxybutyric acid caused a marked depletion in the liver and muscle glycogen, thus suggesting that this compound may disturb phosphorylation.

The present paper shows that the injection of acetoacetate into rabbits reduces the glucose tolerance and the glycogen stored in liver and muscle. Amellin,* a plant product which was isolated by Nath(14) and shown to be highly beneficial in more than 50 clinical cases of diabetes(15-20), restored the glucose tolerance to normal and checked the depletion

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* This is a complex protein-like substance which contains a purine base, a thio sugar, and a phosphate group.

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15. Nath, M. C., and Banerjee, S. R., *An. Biochem. Exp. Med.*, 1943, v3, 107.
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in liver and muscle glycogen. Comparative studies with insulin were also carried out.

*Experimental.* Twenty healthy rabbits, each weighing approximately 2 kg and having fasting blood sugar levels ranging between 106-116 mg per 100 cc, were selected. They were kept on a diet of germinated green gram *(Phaseolus mungo)* and green grass during the whole experimental period. The sodium salt of acetoacetic acid, which was prepared in the usual way, was given intraperitoneally. Since we previously(4) observed that the hyperglycemia induced by injecting the intermediary fat metabolites did not persist unless the successive dose was increased, the animals in our present experiment were given increasing doses of sodium acetoacetate, beginning with a daily dose of 50 mg/kg of body weight. The dose was gradually increased up to 300 mg/kg of body weight, as shown in the tables. The glucose tolerance test was carried out after fasting the animals for about 18 hours and the glucose was given subcutaneously in doses of 1.75 g/kg body weight. The method of Hagedorn and Jensen(21) was used for the estimation of the blood sugar. Some of the experimental animals were killed periodically and the glycogen stored in liver and leg muscle was determined according to the method of Good, Kramer and Somogyi(22). After 90 days, the experimental rabbits were divided into 3 groups. The animals in the first group were injected with sodium acetoacetate in increasing doses as mentioned before. The second group of animals was given 'Amellin' subcutaneously, 12 mg/kg body weight per day along with sodium acetoacetate as in Group 1, while the third group were given insulin subcutaneously, 0.5 unit/kg/day plus sodium acetoacetate. The comparative effects of these 2 drugs on glucose tolerance and glycogen storage were studied for an additional 40 days. After 130 days the dose of insulin was increased to 0.75 unit/kg body weight in some animals in order to see if this would facilitate glucose utilization.

*Results.* At the beginning of the experiment, prior to the injection of acetoacetate, the fasting blood sugar level ranged between 106 and 116 mg/100 cc. When a glucose tolerance test was performed the maximum blood sugar value was reached within a half hour and the blood sugar returned to the initial level within 2½ hours (Table I).

Following the administration of acetoacetate in doses of 50 mg/kg for 10 days, we found an indication of hypoglycemia in all the animals, as reported previously from this laboratory. After 30 days of injection, all the animals had passed over the hypoglycemic stage and showed definite indications of a fasting hyperglycemia. In most cases the blood sugar values during the glucose tolerance test did not return to the initial values within 2½ hours. After 50 days of acetoacetate injection on the shape of the glucose tolerance curve changed so that the maximum B. S. value was reached after 1½ hours and the B. S. values after 3½ hours are, in most cases, far greater than the initial ones. The curves were typically diabetic in character. By the 70th and 90th day the hyperglycemia increased further and there was a progressive decrease in glucose tolerance. At 90 days some of the initial B. S. values exceeded 190 and the average percentage increase of B.S. during the glucose tolerance test increased to 68% (normal = 35%). Average glucose tolerance curves at different periods of injection are represented in Table I. Tidwell and Axelrod(23), studied the effect of acetoacetate in rats for a period of only 4 weeks. During this time they observed hypoglycemia, but they did not observe the second hyperglycemic phase. This may be due to their failure to continue

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21. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, v135, 46.

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23. Tidwell, H. C., and Axelrod, H. E., *J. Biol. Chem.*, 1948, v172, 179.

TABLE I. Average Glucose Tolerance of Rabbits Injected with Sodium Acetoacetate (Glucose Injected SQ. in Doses 1.75 g/kg Body Weight).

| Days of acetoacetate* inj. | No. of rabbits used for glucose tolerance | Blood sugar in mg/100 cc blood | | | | | | | | | | Avg % increase in blood sugar during glucose tolerance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Initial | | ½ hr | | 1½ hr | | 2½ hr | | 3½ hr | | |
| | | Avg | σ | Avg | σ | Avg | σ | Avg | σ | Avg | σ | |
| 0 | 20 | 112 | 2.7 | 229 | 21.1 | 153 | 13.9 | 112 | 4.0 | 113 | 2.5 | 35.2 |
| 10 | 19† | 91 | 6.6 | 193 | 12.2 | 175 | 8.5 | 89 | 7.5 | 92 | 7.5 | 51.3 |
| 30 | 18 | 141 | 9.0 | 250 | 18.0 | 217 | 21.6 | 178 | 25.6 | 149 | 13.7 | 40.6 |
| 50 | 17 | 156 | 13.8 | 246 | 17.3 | 302 | 15.3 | 222 | 24.0 | 204 | 10.1 | 56.6 |
| 70 | 13† | 165 | 18.2 | 261 | 11.8 | 335 | 26.9 | 255 | 20.8 | 234 | 25.6 | 64.8 |
| 90 | 10 | 171 | 20.7 | 283 | 22.3 | 347 | 26.2 | 274 | 28.8 | 250 | 24.9 | 68.3 |

* Daily dose of acetoacetate inj.: 1st to 10th day, 50 mg/kg; 11th to 30th day, 100 mg/kg; 31st to 50th day, 150 mg/kg; 51st to 70th day, 225 mg/kg; 71st to 90th day, 250 mg/kg; 91st to 110th day, 275 mg/kg; 111th day to 130th day, 300 mg/kg; (131st day onwards increase by 25 mg/kg every 20 days).

$\sigma$ = Stand. dev.

† One rabbit died before the 10th day and 4 died between the 50th and 70th day. The other animals were killed for glycogen studies.

the study past 30 days or to a species difference in the response to acetoacetate. Nath *et al.*(24) have shown that acetoacetate can stimulate the biosynthesis of vitamin C in rats far more than in rabbits. As vitamin C is known to be highly beneficial in improving glucose metabolism(25) it might be expected that the hyperglycemic phase in rabbits would be more pronounced than is the case in rats.

*Comparative effect of Amellin and insulin on acetoacetate induced diabetes.* After 90 days when all the animals showed definite hyperglycemia and decreased glucose tolerance, the effects of insulin and Amellin were studied. Amellin was injected into 6 fasting animals in varying doses (20 mg, 30 mg and 50 mg/kg body weight) and its effect on the blood sugar was determined during the subsequent 3½ hours. The animals were fed for 4 hours and then allowed to fast for 16 hours. The same experiment was repeated on the 2nd day and the fasting 48 hour blood sugar level was also determined. Parallel studies with insulin (0.5 unit per kg) were carried out in four other animals treated in the same way. The results are shown in Table II. Six of these animals were given Amellin (10 mg/kg body weight/day) and 4 rabbits were injected with insulin (0.5 unit/kg/day) for 20 days; the injection of acetoacetate was continued in the doses stated previously. At the end of this period a glucose tolerance was performed. After an additional 20 days of combined acetoacetate plus Amellin or insulin injections the glucose tolerance was repeated. At this time the insulin dose was increased from 0.5 unit to 0.75 unit/kg and 3 rabbits received this increased insulin dose for 7 days after which time a glucose tolerance test was again performed. The results are shown in Table III.

It will be noted that Amellin treatment not only lowered the fasting blood sugar value but it also improved the glucose tolerance. Although insulin lowered the blood sugar temporarily it did not produce a permanent decrease in the fasting blood sugar level as did Amellin, for in the 3 rabbits that had received insulin for 47 days the initial blood sugar values ranged between 198 and 210 mg/100 cc 24 hours after the injection of insulin plus glucose.

*Depletion in the glycogen storage and its restoration.* The animals were killed periodically, *i.e.* after 10, 30, 90 and 130 days and the glycogen contents of the liver and muscle were determined in the usual way. The following table indicates the enormous loss of glycogen, as compared to normal animals. These data are in agreement with those observed in rats under similar condi-

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25. Himsworth, H. P., *Lancet*, 1936, v1, 127.

TABLE II. Immediate Effect of Amellin and Insulin on B.S. Values of Animals with Hyperglycemia Induced by Acetoacetate Injection for 90 Days. (Dose on the 90th day 250 mg/kg).

| | | | Blood sugar (avg values) mg/100 cc blood | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment | | | First day After | | | | | Second day After | | | | |
| No. rabbits | Substance | Dose inj. (mg/kg) | Initial (fasting) | ½ hr | 1½ hr | 2½ hr | 3½ hr | Initial (fasting) | ½ hr | 1½ hr | 2½ hr | 3½ hr | 3rd day initial (fasting) |
| 2 | Amellin | 20 | 154 ± 18 | 148 ± 17 | 175 ± 12 | 126 ± 6 | 115 ± 5 | 161 ± 7 | 119 ± 0.5 | 114 ± 1 | 110 ± 2 | 107 ± 1 | 126 ± 7 |
| 2 | | 30 | 187 ± 1 | 181 ± 0.5 | 187 ± 1 | 206 ± 2 | 157 ± 3 | 184 ± 2 | 134 ± 1 | 132 ± 3 | 125 ± 3 | 122 ± 3 | 115 ± 3 |
| 2 | | 50 | 205 ± 9 | 198 ± 2 | 165 ± 0 | 138 ± 2 | 101 ± 11 | 158 ± 32 | 155 ± 43 | 117 ± 9 | 109 ± 5 | 104 ± 7 | 123 ± 7 |
| 4 | Insulin | .5 U | 186 ± 7 | 126 ± 6 | 145 ± 6 | 157 ± 8 | 157 ± 15 | 176 ± 16 | 116 ± 9 | 139 ± 13 | 146 ± 5 | 164 ± 6 | 178 ± 13 |

TABLE III. Effect of Chronic Amellin and Insulin Treatment on Hyperglycemia and Abnormal Glucose Tolerance Induced by Acetoacetate* Injections.

| | | Treatment | | | Blood sugar mg/100 cc blood | | | | | | | | | | Avg % increase in blood sugar during glucose tolerance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days of acetoacetate inj. | No. of rabbits | Substance | Dose per day/kg | Days of treatment | Initial Avg | Initial σ | ½ hr Avg | ½ hr σ | 1½ hr Avg | 1½ hr σ | 2½ hr Avg | 2½ hr σ | 3½ hr Avg | 3½ hr σ | |
| 90 | 10 | 0 | 0 | 0 | 171 | 21 | 283 | 22 | 347 | 26 | 274 | 29 | 250 | 24 | 68.3 |
| 110 | 2 | 0 | 0 | 0 | 168 | 7 | 195 | 9 | 317 | 7 | 274 | 3 | 266 | 2 | 56.5 |
| 110 | 6 | Amellin | 10 mg | 20 | 132 | 18 | 204 | 53 | 152 | 27 | 132 | 34 | 102 | 29 | 12.1 |
| 110 | 4 | Insulin | .5 U | 20 | 114 | 21 | 170 | 18 | 109 | 4 | 97 | 8 | 108 | 13 | 6.1 |
| 130 | 2 | 0 | 0 | 0 | 174 | 3 | 195 | 0 | 389 | 5 | 290 | 5 | 283 | 2 | 66.1 |
| 130 | 5 | Amellin | 10 mg + 12 mg | 20 + 20 | 105 | 9 | 173 | 26 | 112 | 4 | 107 | 6 | 108 | 3 | 19.0 |
| 130 | 3 | Insulin | .5 U | 40 | 182 | 33 | 264 | 13 | 235 | 29 | 162 | 76 | 157 | 76 | 12.6 |
| 137 | 3 | " | .5 +.75 | 40 +7 | 213 | 13 | 280 | 9 | 313 | 9 | 122 | 14 | 139 | 19 | 0.5 |

* For dose of acetoacetate see Table I.

TABLE IV. Glycogen Content of Liver and Muscle Taken from Rabbits Injected with Acetoacetate and Treated with Amellin (10-12 mg/kg/day) or Insulin (.5-75 U/kg).

| No. of rabbits | Days of acetoacetate* inj. | Treatment | Avg body wt, g | Glycogen mg/100 g tissue | |
|---|---|---|---|---|---|
| | | | | Liver | Leg muscle |
| 2 | 0 | 0 | 1400 | 234.3 ± 12.4 | 138.8 ± 11.3 |
| 1 | 10 | 0 | 1620 | 175.2 | 60.5 |
| 1 | 30 | 0 | 1550 | 145.7 | 43.4 |
| 1 | 90 | 0 | 1100 | 86.0 | 37.5 |
| 2 | 130 | 0 | 2000 | 82.0 ± 5.2 | 37.0 ± 4 |
| 2 | 150-151 | Amellin† | 1295 | 230 ± 20 | 57.8 ± 25 |
| 2 | 150-151 | Insulin† | 1470 | 154 ± 14 | 48.5 ± 20 |

* Dose as in Table I.
† Administered from the 91st to the 150th day.

tions(12,13). The effects of Amellin and insulin on the restoration of liver and muscle glycogen are shown in Table IV. It will be noted that the repeated administration of Amellin to rabbits receiving acetoacetate restored the liver glycogen almost completely and the muscle glycogen to a lesser extent. Insulin was less effective in restoring liver and muscle glycogen than was Amellin. Although all the animals treated with acetoacetate became hyperglycemic and showed typical diabetic glucose tolerance curves and glycogen depletion, none showed glycosuria at the end of the 130 day experimental period. Injections were therefore continued in some animals to see if they could be made glycosuric on prolonged treatment. After 140 days of injection, glucose was injected for a tolerance test (1.75 m kg) and one rabbit showed glycosuria (0.4% glucose in the 24 hour sample). The treatment with acetoacetate was continued and on the 160th day the same rabbit developed a spontaneous mild glycosuria (0.1% sugar in 24 hour sample). The animals which were killed periodically for the estimation of glycogen, were also studied morphologically. Tumors were found in the livers of all animals receiving only acetoacetate; their size varied proportionately with the period of injection. The kidneys also showed signs of progressive damage. No tumors were seen in the animals treated simultaneously with acetoacetate plus either Amellin or insulin for about 40 days. The kidneys of these animals were also found to be more or less normal.

It has been observed by Joslin(26) that human diabetes is frequently preceded by a period of obesity. Foglia(27) found that after the removal of 80 to 95% of the pancreas in rats there is an initial period of obesity with no manifest signs of diabetes. This stage is then followed by a period of alimentary hyperglycemia which, in turn, is followed by fasting hyperglycemia and glycosuria and finally a period of ketosis and death. This progression of diabetes in subtotal pancreatectomized rats parallels in many ways the development of human diabetes(27). As similar stages have also been observed in the experimental animals reported here, it is possible that this progression of diabetes induced by intermediary fat metabolites may represent an experimental diabetes which closely parallels the development of human diabetes.

The hyperglycemic effect of a high fat and low carbohydrate diet as observed by Himsworth(28) can also be accounted for in the light of these findings.

The beneficial effects of Amellin in animals with diabetes induced by ketone body injection might be due to either its ketolytic effect as observed by Nath and Habibul Islam (29) or to its action on enzyme systems. Amellin, which has been found to contain a thio group, may also exert its protective

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27. Foglia, V. G., *Rev. Soc. Argent. de Biol.*, 1944, v20, 21.

28. Himsworth, H. P., *Clin. Sc.*, 1935-36, v2, 117.

29. Nath, M. C., and Habibul Islam, A. H. M., *Ind. Jour. Med. Res.*, 1948, v36, 61.

influence like glutathione, which in large doses has been found by Lazarow(30,31) to completely protect rats against the development of alloxan diabetes.

*Summary.* 1. The repeated daily injection of sodium acetoacetate in progressively increasing doses (beginning at 50 mg/kg body weight) caused a progressive hyperglycemia in rabbits after an initial hypoglycemic stage. 2. The glucose tolerance of these animals was decreased after 30 days of injection and the tolerance curve taken at 30, 50, 70, 90, 110 and 130 days became progressively more diabetic. 3. The shape of the tolerance curves changed considerably on prolonged treatment with acetoacetate and in most cases the blood sugar levels at 3½ hours were far greater than the pre-injection level. 4. The glycogen content of liver and muscle is gradually depleted, so that after 130 days of injection it was decreased 64.9% and 73.4% respectively. 5. When Amellin was injected daily for 40 days in doses of 10-12 mg/kg into these hyperglycemia animals it reduced the blood sugar to 105 mg/100 cc (normal value). Insulin, although it relieved the fasting hyperglycemia initially, had little effect on the fasting hyperglycemia in later stages. Increasing the insulin dose leads to death. 6. The glucose tolerance of the animals treated with sodium acetoacetate became absolutely normal after Amellin had been administered for 40 days, whereas the tolerance of similar animals treated with insulin was not normal at 40 days.

*Addendum.* After this paper was presented for publication Griffiths(32) reported that uric acid produced diabetes in rabbits only when their blood glutathione was lowered to about half the normal value. Acetoacetate injection has recently been found in this laboratory to lower blood glutathione considerably(33). These observations may help to explain how acetoacetate favors the development of hyperglycemia and how Amellin acts as a protective substance.

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31. Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 441.

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## Alpha Tocopherol in Treatment of Alloxan Diabetes in Dogs.* (18188)

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Reports have appeared in the literature on the use of alpha tocopherol, in treatment of diabetes(1,2). Recent publications reviewing therapy of diabetes mellitus have invariably made a plea for fundamental controlled work to be done on the use of alpha tocopherol in the treatment of diabetes mellitus(3). Therefore, it was considered worth while to study the effect of alpha tocopherol on alloxan diabetes in the dog.

*Method of study.* Ten dogs were rendered diabetic by a freshly prepared 5% solution of alloxan administered in intravenous doses of 65 to 75 mg per kilo body weight. The dogs were then divided into 2 groups, 7 comprising the experimental group, 3 the control group. The experimental dogs were treated with diet, insulin and alpha tocopherol. The control

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1. Vogelsang, Arthur, *Medical Record,* June-July 1948, p. 1.

2. Butturini, Ugo, *Gior. di Clin. med.* 1945, v26, 90.

3. Joslin, E. P., *J.A.M.A.*, 1949, v140, 581.