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FURTHER OBSERVATIONS ON THE EFFECTS OF ALLOXAN ON THE PANCREATIC ISLETS

By J. SHAW DUNN, E. DUFFY, M. K. GILMOUR, J. KIRKPATRICK and N. G. B. McLETCHIE
From the Pathological Department of the University and Western Infirmary, Glasgow

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In the accounts which have been given [Dunn, McLetchie & Sheehan, 1943; Dunn, Kirkpatrick, McLetchie & Telfer, 1943] of the finding of necrosis of the pancreatic islets after intravenous injection of alloxan in rabbits, it was pointed out that this lesion came to light only because of the unexpected deaths at 12–48 hr. of certain of the animals so treated. Later it was recognized that such deaths were preceded by hypoglycaemia and hypothermia, and sometimes by convulsions as had been observed by Jacobs [1937]. The doses which gave fatal results were usually relatively large, 200–300 mg./kg. body weight, but it was known, from experiments done prior to finding the islet lesions, that similar deaths might occur, though rarely, after a much smaller dose, in one case 25 mg./kg. On the other hand, some rabbits had survived for more than 48 hr. doses of 300 and even 500 mg./kg., given on 1 day, without it being realized from ordinary observation that they had passed through a critical phase. As the pancreas was not examined in these animals the condition of the islets was not ascertained, nor was any alteration of the blood sugar suspected. Once the lesion of the islets had been found it appeared desirable that further information should be obtained as to the conditions of its occurrence and its pathogenesis, and it has been possible to carry out some experiments with this in view. These experiments have combined general observations on the animals and estimations of blood sugar with histological examination of the pancreas, and have been intended to find answers to three main questions: (1) the frequency of occurrence of islet necrosis after single intravenous doses of alloxan such as have been known to produce it; (2) the rate of development of necrosis from the earliest stages, and (3) the effects, if any, of smaller doses.

Experiments

The rabbits used were of various breeds. They were kept in metabolism cages and their usual daily food was 150 g. each of oats and bran, mixed with 200 c.c. water. About 150 g. cabbage was also given daily with rare lapses due

to difficulties of supply. The output of urine was measured: in testing for sugar Benedict's reagent was used. The blood sugar was estimated by the Folin and Wu method. For fixation of all tissues, except the pancreas, Bouin's solution gave satisfactory results. For the pancreas the best results were obtained with Helly's formol-Zenker solution (5–8 hr.), but even with this fixative it proved more difficult to secure standard fixation of the soft and rather loose tissue of the rabbit pancreas, especially from freshly killed animals, than of any of the other organs. Under- and over-fixation are both undesirable and the differing thickness of the various parts of the pancreas of different rabbits may render it difficult to avoid either of these.

Stains used have been Mayer's acid haemalum and eosin, methylene-blue eosin, and modifications of Bensley's fuchsin—aniline-blue—orange method [Warren, 1938] as follows:

1. Acid fuchsin	20 g.	
Aniline oil	5 c.c.	
Distilled water	100 c.c.	10 min.
2. Wash rapidly in distilled water		
3. 1 % phosphomolybdic acid		10 min.
4. Stain for ½–1 hr. or more in:		
Soluble blue	0·5 g.	
Orange G	2·0 g.	
Oxalic acid	2·0 g.*	
Distilled water	100 c.c.	
5. Differentiate in methylated spirit		2–4 min.
6. Dehydrate and mount		

* Or 8 c.c. glacial acetic acid.

Stage 4 should be continued until the acinar tissue is blue and only the α-cells and red corpuscles are strongly red. Differentiation renders normal β-cells blue to low-power examination.

The special feature of this method which has been relied upon for the present work, is its strong selective staining of α-cells in orange-red or red. This is so distinctive, even in acutely damaged islets, that cells not so stained can clearly be placed in another category and we have reckoned the latter as β-cells. In live-fixed tissue the stainable material in the α-cells may only rarely appear as distinct granules. The method renders the granules of normal β-cells blue or blue-violet, but so far this result has not been obtained with certainty in acutely damaged cells, which tend to take the orange.

(1) *Effects of single large doses of alloxan. Exps.* 1–9

The pancreas was examined in nine rabbits after single large doses of alloxan alone. During the first few hours these animals were quiet and inclined to be stationary, but were quite strong when handled and some would eat cabbage or carrot. No. 6 developed hypoglycaemic convulsions at 5½ hr. and no. 7 at 6½ hr. and had to be resuscitated by glucose *per os*. Fig. 1 shows the movements

of blood sugar in nos. 4–9 for 4–6½ hr. After the last recorded reading, glucose was given by stomach tube to all except no. 5 in order to prevent death from hypoglycaemia during the night; and this was successful except in no. 6. No. 5, which received no glucose, appeared perfectly normal until it was killed

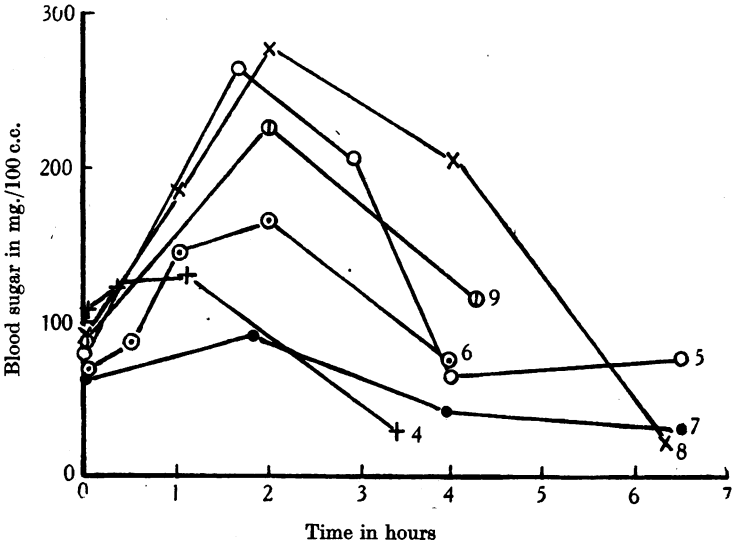


Fig. 1.

at 24 hr. Nos. 4, 6 and 8 had been given water only on the day before injection; the others were fed normally. Nos. 4 and 6, which were starved, had comparatively slight initial rises of blood sugar and developed hypoglycaemia early: no. 8 had a high initial rise but was hypoglycaemic at 6 hr. Against this,

TABLE 1

No.	Alloxan mg./kg.	Result	State of islets
1	300	Killed at 9 hr.	Necrosis in all
2	300	Killed at 12 hr.	Necrosis in all
3	300	Died at 18 hr.	Necrosis in all
4	300	Died at 33 hr.	Necrosis in all
5	300	Killed at 24 hr.	Necrosis in all
6	200	Died at 15 hr.	Necrosis in all
7	200	Glycosuria: killed at 25 days	Almost complete disappearance
8	200	Glycosuria: died at 10 days	Almost complete disappearance
9	200	Glycosuria 20 days: killed at 50 days	Loss of some islets and disorganization of others with regeneration

no. 7, which was fed normally, had no initial rise of blood sugar and developed hypoglycaemic convulsions at 6½ hr. Other significant data are given in Table 1. In four other rabbits which had doses of 200 mg./kg. there was glycosuria continuing for 18 days up to at least 3 months. From these results it appears justifiable to conclude that destruction of islet cells, which may be

very extensive or almost complete, always results from intravenous injection of at least 200 mg./kg. of alloxan, but unless hypoglycaemic convulsions develop it is possible to have little indication from general observation that an animal is ill.

The histological appearances of damaged islet cells in the acute stages of such experiments from 9 hr. onwards are those of coagulative necrosis and have already been described. By the modified Bensley method the majority of the necrosed cells stain pale orange, though fine blue granules are sometimes seen in them. They are evidently β-cells and these appear to be universally affected, though it would be impossible to say that no cell of this type survives in any islet. The strongly eosinophile cells which may be observed in many islets, usually at the periphery, stain characteristically as α-cells. Many of these exhibit the features of live cells with normal nuclei but with swollen bodies packed with the specific granules. Some identifiable α-cells are obviously necrosed with shrunken pyknotic nuclei so that their immunity to the action of the agent is not absolute but relative.

(2) *Early stages of development of the islet lesions. Exps.* 10–15

Six rabbits received 300 mg./kg. of alloxan intravenously and were killed for examination at $1\frac{1}{4}$–5 hr. The movements of blood sugar in five of these are shown in Fig. 2. In the accounts of these experiments only the state of the islets is described. In all cases the acinar tissue of the pancreas was normal, but eosinophile material of colloid appearance was sometimes seen in the smaller ducts. The thyroids and suprarenals showed no changes.

Exp. 10. Wt. 1900 g., killed at $1\frac{1}{4}$ hr. On low-power examination the islets had an open cribriform appearance in excess of anything seen normally: with the high power this was confirmed in that the cell ribbons were separated from one another by a space, fully the width of a cell: scanty fine basophile particles were frequently present in this space (Pl. 1, fig. 4). With haemalum and eosin the cell bodies were of normal appearance, but in the majority of the central cells, no doubt all β-cells, the nuclei were definitely hyperchromatic though retaining their normal size and outline and the appearance of a reticulum. With Bensley's stain the α-cells, which normally are relatively scanty and may be from 0 to 8 in any islet section, were of normal appearance and had normal nuclei. The β-cells showed no depletion of blue-stained material in their cytoplasm but distinct granules were not identified.

Exp. 11. Wt. 1900 g., killed at $1\frac{1}{2}$ hr. The islets showed less degrees of the changes seen in Exp. 10: hyperchromasia of nuclei was less and affected fewer cells.

Exp. 12. Wt. 1940 g., killed at 2 hr. The appearances in the islets were as in Exp. 10 and not more advanced.

Exp. 13. Wt. 2000 g., killed at 3 hr. The islets showed well-marked changes: hyperchromasia of nuclei in the central cells was intense, sometimes obliterating the appearance of a reticulum, but there was no karyorrhexis. The cytoplasm of these cells stained more homogeneously with eosin than at the earlier stages or in normal cells. In addition, some cells were dislocated from attachment to their fellows, and while some of these retained their quadrate

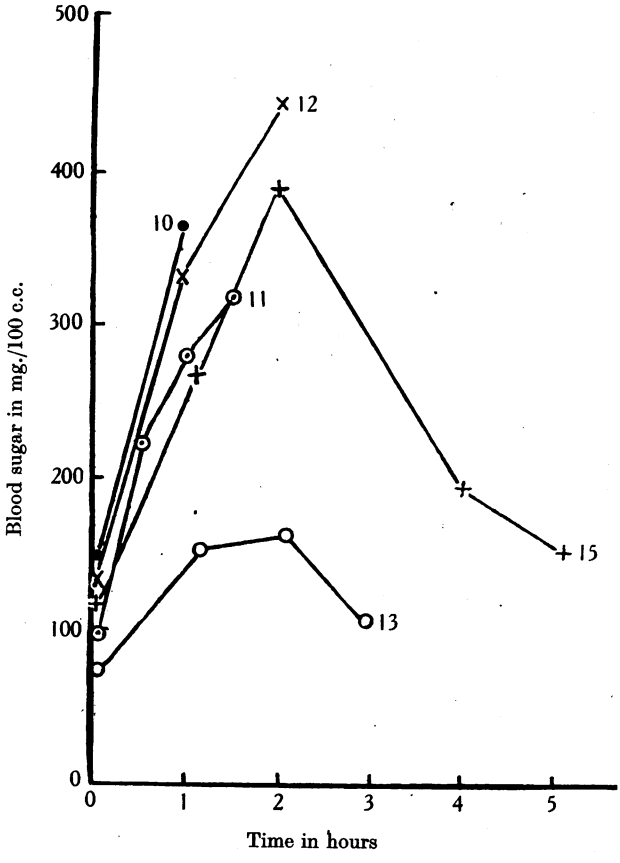


Fig. 2.

or polygonal shape a few had become rounded off and had paler stained cytoplasm. The strongly eosinophile α-cells were quite unusually prominent, most being peripheral but some central in the islets. In the majority of the α-cells the nuclei were normal, but in a few, with swollen rounded bodies, the nuclei were shrunken and pyknotic. Bensley's stain gave much more definitive appearances. The brightly stained α-cells were swollen to twice their normal size or more, and had usually become rounded in shape. They appeared more numerous than normally and sometimes formed an almost continuous ring of

up to a dozen cells at the periphery of an islet. The appearance of increase in number was difficult to account for except by the actual increase in size: no mitoses were seen in them. The bodies of the β-cells mostly stained heavily and tended to retain some of the orange; granules could not be seen. Some of the dislocated cells had a washed-out appearance with some granular substance remaining, but it could not be decided that this was specific granulation.

Exp. 14. Wt. 1900 g., killed at 3 hr. 40 min. The islet changes were of the same kind as in Exp. 13 but less severe. In the majority of the cells the nuclei were hyperchromatic but with recognizable reticulum: there was no karyorrhexis. Only a few cells were dislocated. The cytoplasm of the β-cells stained rather more homogeneously than normally and only a few had a washed-out appearance. With Bensley's stain the α-cells were not enlarged or conspicuous.

Exp. 15. Wt. 2000 g., killed at 5 hr. The β-cells here, as in Exp. 13, showed intense nuclear hyperchromasia and homogeneous staining of their cytoplasm with loss of texture suggesting early necrosis. Only a few cells were dislocated (Pl. 1, fig. 5). By Bensley's stain the α-cells were enlarged and rounded as in Exp. 13, but they appeared only slightly increased in number. None of the α-cells had hyperchromatic nuclei nor were mitoses seen in them.

(3) *Effects of smaller doses. Exps.* 16–24

The dose given to these animals was 50 mg./kg., and this appeared to have little or no effect on their well-being, as they moved about and fed normally afterwards. The movements of blood sugar in nos. 16–23 are shown in Fig. 3. The initial rise was not very high in any animal and no hypoglycaemic phase was noted. In nos. 18, 19 and 21 the blood-sugar level remained unaltered throughout. Nos. 16–20 were killed at 24 hr.; nos. 21–24 at 48 hr. No. 24 had a second dose at 24 hr.

Exp. 16. Wt. 3000 g., diet ordinary. Sugar was present in the urine next morning. Killed at 24 hr. Though apparently in perfect health and eating full rations, this animal was found to have an early infection of pseudo-tuberculosis in the lymphoid tissue of the intestine. All the islets were markedly changed. In almost all there was an interrupted ring of swollen, brightly eosinophile cells at the periphery with sometimes a few centrally. These were identifiable as α-cells by Bensley's stain. In the centres the β-cells were almost all rounded off and slightly swollen: their nuclei were pyknotic, yet few showed karyorrhexis or karyolysis: their bodies generally stained pale with eosin and in some the stainable substance appeared washed-out (Pl. 1, fig. 6). With Bensley's stain fine granules could be identified in the β-cells, usually numerous, but in some, corresponding to those described as washed out, they were very scanty, giving the cell bodies an empty appearance.

Exp. 17. Wt. 2300 g., water only was given for 24 hr. before injection. The blood sugar rose to 230 mg./100 c.c. at 4½ hr. Glucose 5 g./kg. was given by

stomach tube at 4½ and at 6½ hr. There was slight glycosuria in the morning. Killed at 24 hr. All the islets showed changes of the same kind as in Exp. 16, though somewhat less in degree.

Exp. 18. Wt. 1350 g., received water only for 24 hr. before injection. There was no rise of blood sugar at 2 or at 4 hr., and no glycosuria appeared. Killed at 24 hr. Many of the islets here retained normal appearances. It was only on systematic examination with the high power that any changes were recognized. These affected only about a tenth of the islets and were restricted to dislocation and rounding off of a few β-cells with a washed-out appearance in some of these.

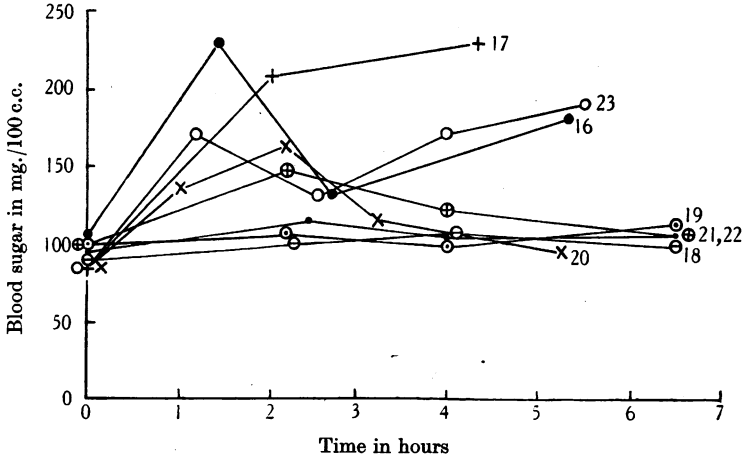


Fig. 3.

Exp. 19. Wt. 1500 g., ordinary diet. There was no rise of blood sugar at 2 or at 4 hr. Killed at 24 hr. The islets appeared practically normal. One or two hyperchromatic nuclei were seen in a few, but it could not be decided that this constituted a definite abnormality.

Exp. 20. Wt. 1150 g., water only was given for 24 hr. before injection. The blood sugar rose to 165 mg./100 c.c. at 2 hr. Glucose 3 g./kg. was given by stomach tube at 6½ hr. and 2 g./kg. at 11½ hr. Sugar was present in the urine next morning and the blood sugar was 320 mg./100 c.c. Killed at 24 hr. Bouin fixation only was available. The majority of the islets appeared normal but in a few there was a washed-out appearance of some of the central cells.

Exp. 21. Wt. 1600 g., water only was given for 24 hr. before injection. The blood sugar rose from 99 to 147 mg./100 c.c. at 2 hr. No glycosuria. Killed at 48 hr. The majority of the islets appeared quite normal even on close inspection, but in one or two there was a group of about a dozen washed-out and crumpled cells with pyknotic nuclei in the centre. Similar appearances affecting fewer cells could be recognized in other islets. There was only a slight degree of swelling and prominence of α-cells in some islets.

Exp. 22. Wt. 1600 g., ordinary diet. There was no rise of blood sugar at 2 hr. Killed at 48 hr. The islets generally appeared normal but on close examination with the high power one or two dislocated cells with shrunken bodies and pyknotic nuclei were seen in a few of them. No mitoses were found.

Exp. 23. Wt. 1150 g., ordinary diet. The blood sugar rose from 98 to 170 mg./100 c.c. at ½ hr. and remained at about that level for 5½ hr., falling to 110 mg. at 10 hr. Next morning it was 120 mg. The animal was killed at 48 hr. On low-power examination the islets were notably altered, the majority being reduced in size and having very irregular ill-defined outlines (Pl. 1, fig. 7). With the high power it was clear that this deformation had resulted from collapse of the centres of the islets due to shrinkage or disappearance of cells, whilst groups of well-preserved cells at the periphery had been pushed inwards. In the centres the remaining cells were variable in appearance: some showed shrunken pyknotic nuclei and greatly shrunken bodies: a few were necrotic with karyorrhexis or karyolysis: others showed a washed-out appearance of their cytoplasm. The majority of the well-preserved peripheral cells were α-cells and these had swollen eosinophile bodies which took the orange-red stain by Bensley's method. Mitoses were seen in peripheral cells in some islets but rarely more than one in any islet (Pl. 1, fig. 8): the category of the dividing cells could not be determined as their cytoplasm was bloated and lacking in specific granulation.

Exp. 24. Wt. 2300 g., full diet with carrots. Doses of 50 mg./kg. given on first and second days: there was practically no rise of blood sugar after the first dose. Killed 48 hr. after first dose. With low power most islets appeared normal but the high power revealed various degrees of damage in about one-fifth: the commonest change was dislocation and rounding off of β-cells; their nuclei were hyperchromatic and the cytoplasm had a washed-out appearance (Pl. 1, fig. 9). In one or two islets there was definite necrosis of a central mass of cells. The α-cells in damaged islets were generally swollen and prominent. No mitoses were found on careful search either in the damaged islets or in the more normal ones.

Discussion

The results of the first nine experiments, and of four others referred to, show that intravenous doses of alloxan of at least 200 mg./kg. in rabbits always cause severe damage to the islets of Langerhans. While this lesion is developing most animals are somewhat depressed and may not eat, and some pass into hypoglycaemic convulsions after 4–24 hr.: death then occurs unless glucose is administered [cf. Jacobs, 1937]. It is possible, however, for a rabbit to survive a dose of 200 mg./kg. for 24 hr. with necrosis in the whole of its islets and without showing very definite outward signs of illness (Exp. 5). This fact, which could hardly have been expected where such a specialized and important tissue was concerned, helps to account for the apparent capriciousness in effect even of

large doses of alloxan: it may also have some significance in relation to the possible occurrence of acute damage of the islets from any cause in the human subject.

It has already been shown that with large doses of the above order the islet cells exhibit the features of coagulative necrosis at nine hours, and the view that by that time they are dead is confirmed by the progress of nucleolysis in animals examined at later periods. In Exps. 10–15 the doses were 300 mg./kg., and it is considered certain that frank necrosis would have been recognizable in the islets had the animals been allowed to live for 9 hr. or longer. Histological examination revealed certain changes from $1\frac{1}{4}$ hr. onwards: the earliest and most definite was hyperchromasia of β-cell nuclei, which preceded any recognizable cytoplasmic change. The hyperchromasia was increased in intensity at 3 and at 5 hr. and was then associated with altered staining of the cytoplasm and with dislocation of some cells. It is impossible to tell by histological examination the earliest stage at which cells are dead, but it seems probable that death had occurred in some cells at 3 hr. when they were separating from their attachments: it may, of course, have occurred earlier. The opening up of channels between the cell ribbons (Pl. 1, figs. 4, 5) at these early stages cannot be interpreted with certainty. Some degree of this may be seen at times in normal islets, though usually the ribbons are closely packed together. Here the condition appeared to be in excess of normal and the spaces contained scanty granular debris suggesting that they had had some fluid content. The appearance is thought to be compatible with a temporary increase in functional activity of the islets which might be in operation immediately after dosage.

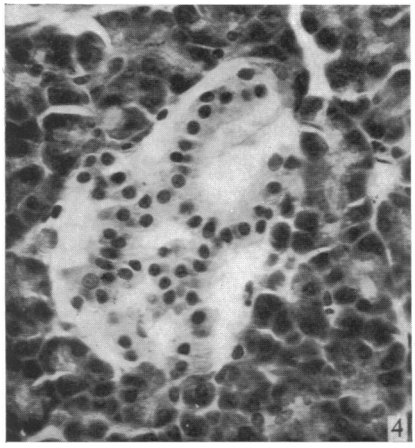
From observation of the general behaviour only of rabbits which received 50 mg./kg. of alloxan (Exps. 16–24), it would not have been suspected that any serious internal lesion had occurred. Examination of the pancreas in these animals gave variable results. In one (no. 19) all the islets appeared normal; in four, nos. 18 and 20 killed at 24 hr., and nos. 21 and 22 killed at 48 hr., the majority were normal and only slight changes were present in the remaining few: these comprised nuclear pyknosis and depletion of cytoplasmic substance in some cells, resulting in a washed-out appearance by ordinary staining. The focal distribution of these slight lesions, notable also in Exp. 24, may possibly be related to the state of activity of individual islets during the period when the agent was having effect. In contrast with these slight changes, there were quite pronounced alterations in the islets in nos. 16 and 17, examined at 24 hr., and in no. 23, examined at 48 hr. In these the β-cells were much changed: they were rarely frankly necrotic but their outlines were rounded off and their nuclei hyperchromatic, while the granular substance in their cytoplasm was much depleted. With the special stain great diminution of the fine β-granules was notable in many cells. By 48 hr. most of these cells had shrivelled or

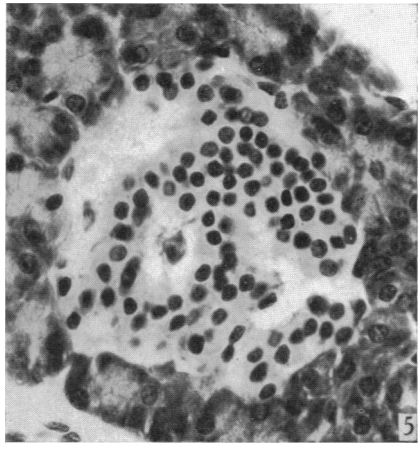
collapsed, taking up little space, so that the islets were small with deformed outlines. These cellular changes were markedly in contrast with those of coagulative necrosis and karyolysis seen at the same periods after large doses, and as they had taken so long to develop it appeared to be a reasonable interpretation that while essentially necrobiotic in character, they were the result of overactivity and exhaustion of the cells after stimulation, rather than of a directly lethal toxic effect.

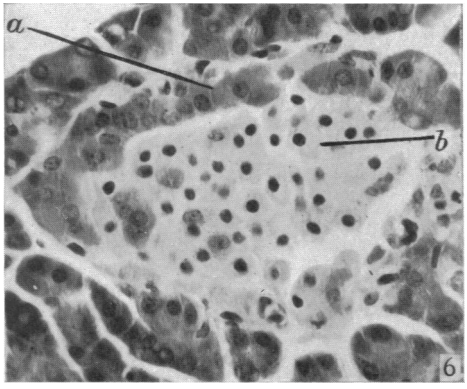
A further interesting and highly suggestive feature in the histological appearances in the islets is the differential effect on α- and β-cells. It had already been noted that in islets necrosed after large doses any surviving cells were situated marginally, and that their bodies were strongly eosinophile. It is now confirmed by special staining that these are α-cells and that their bodies are enlarged to two or three times normal, with evident increase in amount of the specific granule substance. This change may be well established 3 hr. after a large dose and is also quite pronounced 24 hr. after a smaller dose wherever the β-cells are much altered. With this change, which appears to be an active and progressive one, the majority of the α-cells have the appearance of living cells, but some are necrosed. It seems likely that this striking difference in their behaviour is significant of some considerable difference in the respective functions of α- and β-cells, which by this method may become accessible to investigation.

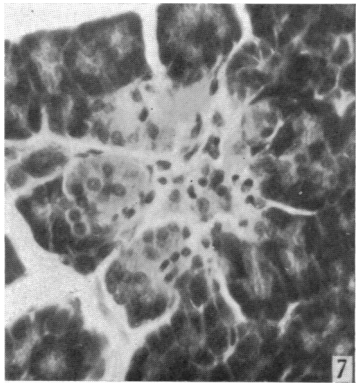
Although the movements of the blood sugar were observed only over the first few hours, a correlation of them with histological changes provides some interesting features. In eleven animals which received large doses (Figs. 1, 2) there was a rise of blood sugar in 2 hr. to 230–440 mg./100 c.c. in seven; and to 130–165 mg. in three, two of which had been starved for 24 hr. In one only, no. 7, which was fed normally, the blood sugar did not rise to 100 mg./100 c.c., but in this animal the initial reading was unusually low and hypoglycaemia set in rapidly. After doses of 50 mg./kg. the blood sugar rose in only five out of eight and the elevation was less than with the large doses: of these five, three were those with well-marked changes in the islets.

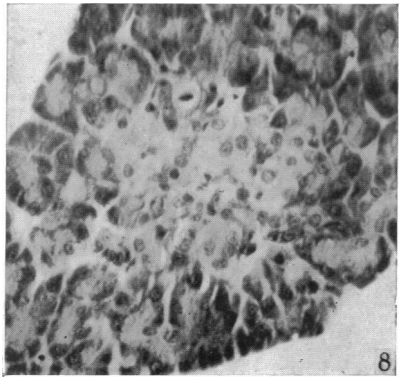
From these observations it may be concluded that a definite initial rise of blood sugar after alloxan probably indicates that damage of islets is occurring. Absence of a rise may mean that damage is slight or nil, but may also be noted even if this is severe. Jacobs, in referring to the transient hyperglycaemia, did not conclude that it was a specific effect of alloxan. The hypoglycaemia which may be evident in 4 hr. appears to be a constant and specific effect if the dose of alloxan is sufficient. Jacobs observed it invariably with doses of 70 mg./kg. and upwards, and we can confirm this from all experiments where the dose was sufficient and suitable examination was made. It will be noted that no hypoglycaemia was observed after doses of 50 mg./kg. (Exps. 16–24).

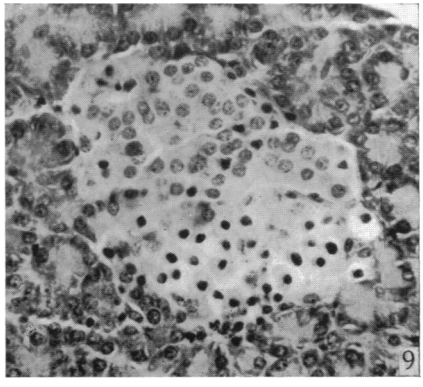












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SUMMARY

1. From observations on fourteen rabbits it has been confirmed that single intravenous doses of alloxan of at least 200 mg./kg. always cause serious damage of the islets of Langerhans, though there may, during the first day, be little outward indication of serious illness. After these large doses definite histological changes can be recognized in the islets after about an hour, and these may be very pronounced at 3 hr. when some β-cells appear to be necrosed.

2. With doses of 50 mg./kg. the β-cells sometimes show degranulation, but little necrosis, at 24 hr.: the appearances are consistent with abnormal increase of function. After these smaller doses islet changes may be only slight and focal in distribution.

3. α-cells are less prone to the necrobiotic changes than β-cells and, from early stages, may be enlarged with apparent increase of granules when the β-cells are necrosed or exhausted: this observation suggests some difference in function of the two types of cell.

4. A temporary hyperglycaemia almost always occurs in the first 2 hr. where there is any damage to the islets.

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EXPLANATION OF PLATE 1

Fig. 4. Exp. 10. Hyperchromasia of nuclei and opening of spaces between cell ribbons. Dose 300 mg./kg.: time 1¼ hr. H. and E. ×400.

Fig. 5. Exp. 15. More intense nuclear hyperchromasia, dislocation of some cells, and spaces formed. Dose 300 mg./kg.: time 5 hr. H. and E. ×480.

Fig. 6. Exp. 16. Rounding off of β-cells with nuclear hyperchromasia and pale staining of cytoplasm: washed-out appearance in some cells (*b*). A border of enlarged α-cells above with normal nuclei and strongly stained bodies (*a*). Dose 50 mg./kg.: time 24 hr. H. and E. ×480.

Fig. 7. Exp. 23. Collapsed and deformed islet with disappearance of most of the central β-cells: clumps of large α-cells peripherally. Dose 50 mg./kg.: time 48 hr. H. and E. ×280.

Fig. 8. Exp. 23. Deformed islet, mainly α-cells. Mitosis is seen in a cell at the upper edge. H. and E. ×280.

Fig. 9. Exp. 24. Nuclear pyknosis and pale cytoplasmic staining of β-cells in lower part of islet, with washed-out appearance in some. Normal cells in upper two-thirds. Dose 50 mg./kg. first and second days: killed at 48 hr. H. and E. ×280.