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Studies on the Mechanism of L-Leucine – and α-Ketoisocaproic Acid – Induced Insulin Release from Perifused Isolated Pancreatic Islets

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Summary. The insulinotropic effects of L-leucine and α-ketoisocaproic acid have been compared in perifused isolated pancreatic islets. In contrast to α-ketoisocaproic acid (10 mM), L-leucine (10 mM) released less insulin in the presence than in the absence of glucose (5 mM). Changes of islet cell metabolism accompanying insulin release were studied by recording the fluorescence of reduced pyridine nucleotides. The traces of L-leucine — or α-ketoisocaproic acid-induced fluorescence increase differed both in the absence and in the presence of glucose (5 mM). When the medium perifusing the islets contained 30 mM L-leucine, α-ketoisocaproic acid (10 mM) still triggered a significant insulin release. These results argue against an indirect action of α-ketoisocaproic acid via transformation to L-leucine. Isocaproic acid (10 mM), L-α-hydroxyisocaproic acid (10 mM) or α-ketoisovaleric acid stimulated no remarkable insulin release, demonstrating that the strong insulinotropic effect of α-ketoisocaproic acid is coupled both to its α-ketogroup and to the length of its carbon chain.

Key words: Pancreatic islets, obese-hyperglycemic mice, perifusion, insulin release, reduced pyridine nucleotides, L-leucine, α-ketoisocaproic acid.

α-Ketoisocaproic acid (KIC), the deaminated metabolite of leucine, elicited an intense insulin release from isolated pancreatic islets [1]. There was some evidence that oxidation of KIC did not provide the signal for stimulation of insulin secretion. In isolated islets of Langerhans KIC, however, enhanced the tissue level and the production of leucine. Therefore the possibility existed that L-leucine was the true trigger of insulin secretion induced by KIC. The aim of the following study was to clarify that point and to gain further insight in the mechanism of L-leucine — and KIC — induced insulin release. Preliminary results of some of the following observations have been reported elsewhere [2, 3].

Materials and Methods

1. Chemicals

Sodium salts of α-ketomonocarboxylic acids (Sigma, St. Louis) were used. Since the preparations contained less than equivalent amounts of sodium, the true sodium content was controlled by atomic absorption and considered when omitting equivalent amounts of sodium chloride from the media. KIC contained less than 0.002% leucine as measured by column chromatography. Sodium succinate, L-leucine, pentobarbital and cycloheximide were from Serva, Heidelberg; bovine serum albumin, crystalline ox insulin (27 U/mg) and ^{125}I-insulin from Farbwerke Hoechst, Hoechst. Guinea-pig anti-insulin serum was prepared in our institute. Stock solutions of L-α-hydroxyisocaproic acid (Sigma, St. Louis), isovaleric and isocaproic acid (Fluka, Buchs) were neutralized with sodium hydroxide. All other substances were obtained from Merck, Darmstadt.

2. Perifusion of Pancreatic Islets

6—8 months old obese-hyperglycemic mice of both sexes were starved for 20—28 h and killed by decapitation. Pancreatic islets were dissected within 25 min at +2° in Krebs-Ringer phosphate buffer supplemented with 3.3 mM glucose [4]. Perifusion at 85 µl/min and 36° was performed as described [1, 5]. The media consisted of Krebs-Ringer bicarbonate buffer. When insulin secretion was measured the medium contained 2 mg/ml bovine serum albumin and was collected in 4 min fractions. When sodium salts of carboxylic acids were added, equimolar amounts of sodium chloride were omitted.

3. Analytical

NAD(P)H-fluorescence [1, 5] and immunologically reactive insulin [6] were measured as described. Ox insulin was used as standard. None of the tested substances influenced the immunoassay. The calcium content of the media was controlled by atomic absorption. By omission of calcium from the media levels lower than 0.1 mEq/l were obtained.

4. Evaluation of Results

In fluorimetric studies typical single experiments are described. Reproducibility was tested by repeating each experiment at least 5 times, using different islets (0.35—0.5 mm, longest diameter). The fluorescence increase induced by 20 mM glucose or 3 mM pentobarbital, served as a reference value. The results described were observed in every single experiment.

Data on insulin release are given as means and standard errors of the mean. The average secretion rates during one exposure period were compared with those from the preceding 12 min control period by the t-test for paired observations. A significant increase was assumed if p was below the 5% level.

Results

10 mM L-leucine stimulated insulin secretion from the substrate-free perifused pancreatic islet with an initial overshoot, accompanied by a twophase increase of the NAD(P)H-fluorescence [1]. To rule out the possibility that the fading of insulin release was brought about by a lack of energy yielding substrates, the responses of islets perifused with media containing 5 mM glucose were examined. It has been shown that 5 mM glucose enhanced the ATP content of pancreatic islets from obese-hyperglycemic mice [7] and only slightly inhibited [U-^{14}C] leucine oxidation by islets [8]. Insulin release triggered by 10 mM L-leucine, however, was significantly ($p < 0.001$; t-test) lower in the presence than in the absence of 5 mM glucose (Table 1, Fig. 1). Simultaneously 5 mM glucose altered the kinetic of the leucine-induced fluorescence increase (Fig. 1). In the presence of 5 mM glucose L-leucine (10 mM) caused a monophase rise of the fluorescence trace, starting 10—20 sec after L-leucine arrived at the islet (Fig. 1).

The typical kinetic of the fluorescence changes in response to 10 mM KIC [1] was also affected by 5 mM glucose (Fig. 2). When the media contained 5 mM glucose, the KIC-induced fluorescence increase did not take place with an initial overshoot. Withdrawal of KIC caused a fluorescence decrease. But the fluorescence intensity remained markedly higher than before addition of KIC (Fig. 2). Glucose (5 mM) did not diminish insulin secretion elicited by 10 mM KIC (Table 1, Fig. 2).

The different effects of glucose upon L-leucine — or KIC-induced insulin release and upon the accompanying fluorescence changes did not support an indirect stimulatory action of KIC via leucine. Furthermore even in the presence of 30 mM L-leucine KIC triggered insulin release, which was not accompanied by a NAD(P)H-fluorescence increase (Fig. 3). 3 mM pentobarbital, however, produced a large fluorescence increase in the presence of 30 mM L-leucine, demonstrating that the lack of fluorimetric response to KIC was not due to low levels of oxidized pyridine nucleotides. It is unlikely that KIC merely acted as a fuel. From data published by Ashcroft *et al.* [9] and Hellman *et al.* [8], one would expect that mouse islets respiring in the presence of glucose (5 mM) and L-leucine (30 mM) are well supplied with substrates yielding metabolic energy. Moreover 10 mM pyruvate elicited no significant ($n = 5$; $p > 0.1$) insulin release by islets perifused with 5 mM glucose and 30 mM L-leucine.

The fluorimetric responses to L-leucine or KIC were not affected when insulin synthesis was inhibited by 1 mM cycloheximide (compare traces a and c of Fig. 4 with the upper traces of Fig. 2 and Fig. 1). But under conditions of inhibited insulin release the fluorescence kinetics caused by L-leucine or KIC were changed. Omission of calcium from the media inhibited insulin secretion induced by leucine [10] or KIC (U. Panten and E. v. Kriegstein, unpublished observations). When glucose-free media were not supplemented with calcium the NAD(P)H-fluorescence remained at a constant increased level during the whole exposure period to KIC (Fig. 4b). In the absence of calcium, L-leucine caused a monophase fluorescence increase (Fig. 4d).

Table 1. *Rates of insulin secretion by perifused isolated pancreatic islets from obese hyperglycemic mice. The average rates of insulin secretion (μU/min/islet) from 24—36 min (control period) and 37—72 min (period of exposure to test substance) of perifusion of islets are indicated*

Test substance	n	Control	Test	p
10 mM L-leucine	7	0.60±0.13	2.29±0.37	<0.001
10 mM L-leucine[a]	17	0.67±0.06	1.13±0.15	<0.001
20 mM L-leucine[a]	4	0.75±0.12	1.83±0.10[b]	<0.01
30 mM L-leucine[a]	8	0.58±0.12	2.63±0.36[c]	<0.001
5 mM α-ketoisocaproic acid	4	0.44±0.09	1.52±0.18	<0.0025
10 mM α-ketoisocaproic acid	8	0.39±0.06	2.15±0.26	<0.001
10 mM α-ketoisocaproic acid[a]	16	0.63±0.05	2.90±0.21	<0.001
10 mM α-ketoisovaleric acid[a]	5	0.77±0.11	1.03±0.18	<0.05
10 mM L-α-hydroxyisocaproic acid[a]	3	0.91±0.15	1.04±0.13	>0.1
10 mM Isovaleric acid[a]	4	0.67±0.10	0.60±0.19	>0.1
10 mM Isovaleric acid	6	0.46±0.07	0.53±0.11	>0.1
10 mM Isocaproic acid	4	0.65±0.10	0.81±0.18	>0.1

[a] = Media contained 5 mM glucose during control and test period.
[b] = Response to 20 mM L-leucine was significantly higher than to 10 mM L-leucine in the presence of 5 mM glucose ($p < 0.01$, t-test).
[c] = Response to 30 mM L-leucine was significantly higher than to 20 mM L-leucine ($p < 0.05$, t-test).

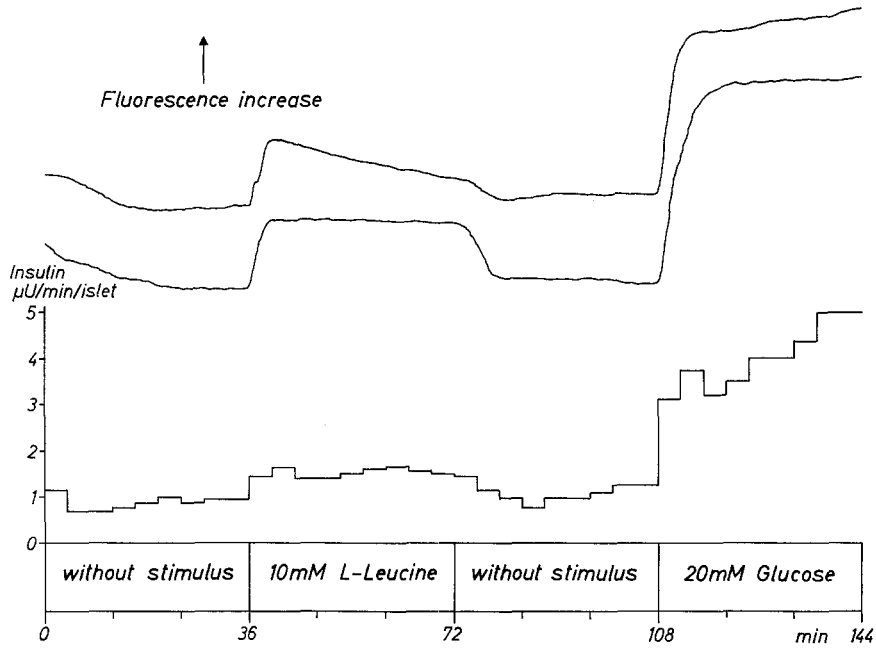

Fig. 1. Effect of L-leucine on insulin secretion of a single islet perifused in the presence of 5 mM glucose from min 0—108 (lowest curve). In a parallel experiment (middle trace) the effect of L-leucine on the NAD(P)H-fluorescence of a single islet perifused in the presence of 5 mM glucose (from min 0—108) is shown. The upper trace shows the effect of L-leucine on the NAD(P)H-fluorescence of a single islet perifused in the absence of glucose (from min 0—108)

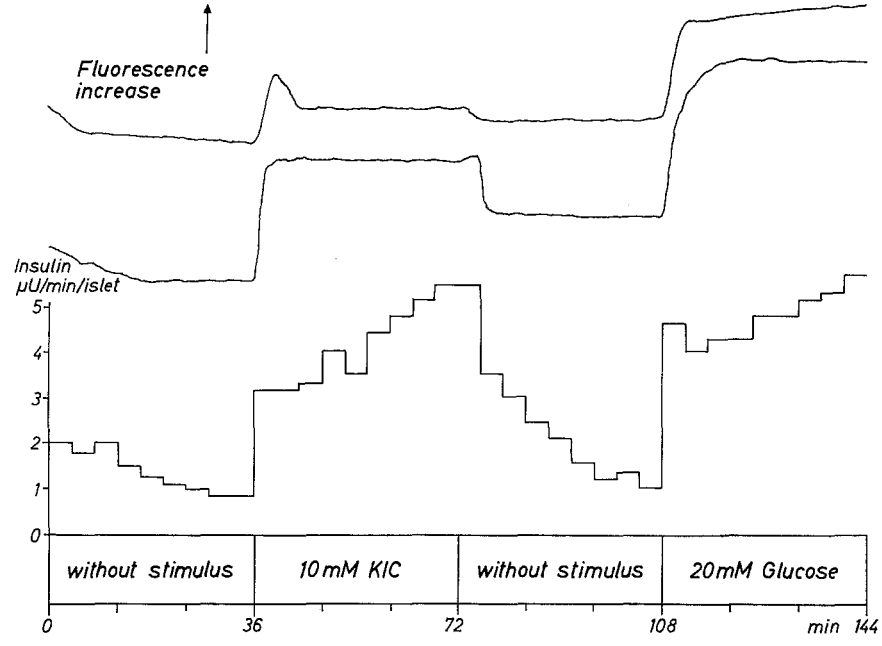

Fig. 2. Effect of KIC on insulin secretion of a single islet perifused in the presence of 5 mM glucose from min 0—108 (lowest curve). In a parallel experiment (middle trace) the effect of KIC on the NAD(P)H-fluorescence of a single islet perifused in the presence of 5 mM glucose (from min 0—108) is shown. The upper trace shows the effect on KIC on the NAD(P)H-fluorescence of a single islet perifused in the absence of glucose (from min 0—108)

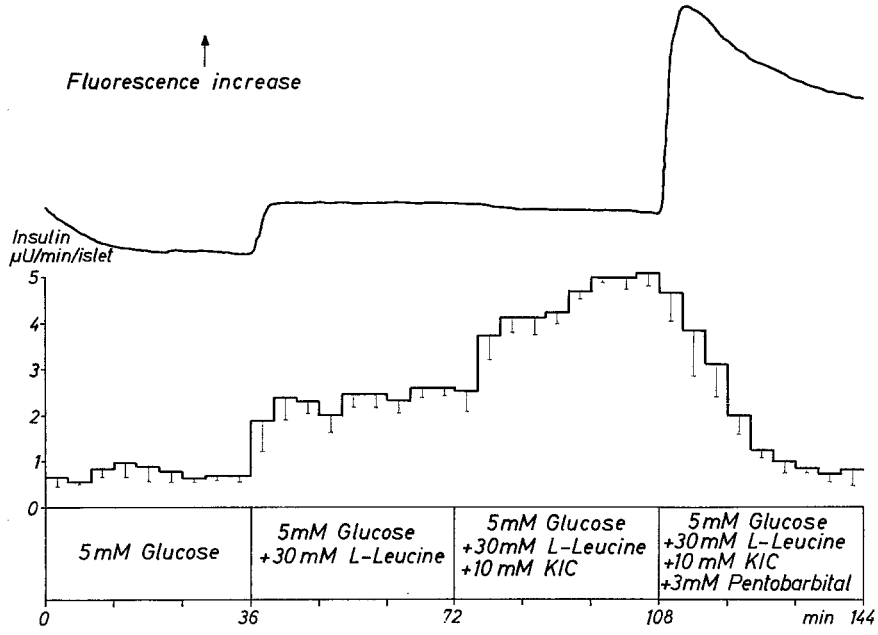

Fig. 3. Effect of KIC on the NAD(P)H-fluorescence trace (upper curve) of a single islet (0.35 mm shortest diameter) perifused with 30 mM L-leucine. The curve represents one of six experiments performed under the same conditions. All experiments gave similar profiles. In parallel experiments the amount of insulin released was immunoassayed (shortest diameter of islets 0.2—0.3 mm). The columns of the secretion profile represent the mean ± s.e.m. of 3 experiments. The average insulin secretion from min 72—108 was significantly higher than that from min 36—72 ($p < 0.005$; t-test)

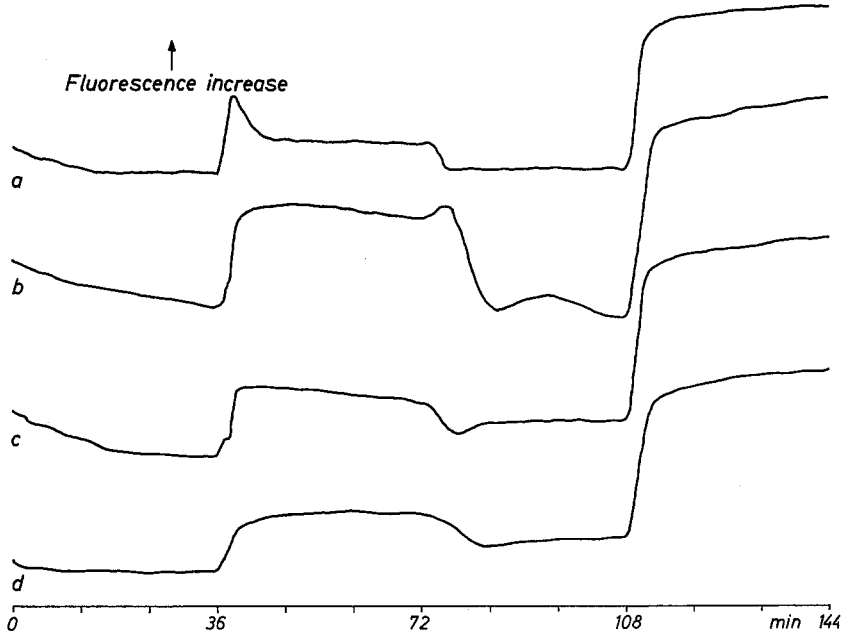

Fig. 4. NAD(P)H-fluorescence traces of single islets. Media contained 10 mM KIC (traces a, b) of 10 mM L-leucine (traces c, d) from min 36—72. Media contained 20 mM glucose (traces a, b, c, d) from min 108—144. Media contained 1 mM cycloheximide (traces a, c) from min 1—144. Media contained no calcium (traces b, d) from min 1—144. No other substrates were added to the media. Each curve represents one of six experiments performed under the same conditions, all of which gave similar profiles

Some substances structurally related to KIC were tested in order to see which parts of the KIC-molecule are essential for the strong stimulatory effect upon insulin release. Isovaleric acid, isocaproic acid and L-α-hydroxyisocaproic acid did not trigger insulin secretion significantly (Table 1). In the presence of 5 mM glucose 10 mM α-ketoisovaleric acid released only a very small amount of insulin (Table 1) accompanied by a small fluorescence increase (about 15% of the increase caused by 20 mM glucose).

Discussion

In the absence of glucose, L-leucine released more insulin from perifused islets than in the presence of a substimulatory (5 mM) glucose concentration (Table 1). Furthermore, Table 1 shows that 5 mM glucose probably shifted the concentration-response curve to higher leucine levels. This enhanced sensitivity to leucine shown by islets perifused without substrate may be caused by removal of glucose-dependent modifiers of insulin release, e.g. endogenous substrates or glucagon.

If leucine produced by transamination of KIC was the true trigger of KIC-induced insulin release, one would expect a greater insulinotropic effect of KIC in the absence than in the presence of glucose. But such an effect of 5 mM glucose was not seen (Table 1). Islets perifused with 30 mM L-leucine probably did not respond to 10 mM KIC with production of leucine. KIC, however, triggered insulin secretion in the presence of 30 mM L-leucine (Fig. 3). These results did not support the concept that the insulinotropic effect of KIC was caused by its transamination derivative leucine.

The NAD(P)H-fluorescence changes accompanying leucine-induced insulin release probably do not reflect changes induced by metabolic degradation of L-leucine. The nonmetabolized analogue to leucine BCH does elicit a fluorescence response almost identical to leucine although it is most probably not utilized by the B-cell [11]. Therefore it is likely that the fluorimetric signals were consequences of events started by the interaction of L-leucine and b-BCH with their receptor site for insulin release. This view was supported by the changed fluorescence trace (Fig. 4), when the stimulatory effect of L-leucine upon insulin release was inhibited by omission of calcium from the media [10]. The finding that fluorescence changes persist in the absence of calcium may indicate that they reflect a cellular response which precedes the secretory process itself.

The striking similarity of leucine and BCH induced fluorescence traces [1, 11], suggests that the latter can be used as fingerprints to identify substances stimulating insulin release by the same mechanism. But during different experimental conditions, KIC caused fluorescence profiles which never resembled the fluorimetric responses to leucine (Figs, 1, 2, 4).

Mitochondrial degradation of KIC probably does not provide the signal for insulin release [1, 12]. From the presented results it cannot be decided to which extent KIC-induced fluorescence increase results from its mitochondrial degradation or from changes of metabolism triggered by reactions of the stimulus-secretion sequence. The lack of a fluorimetric response to KIC in the presence of 30 mM L-leucine (Fig. 3) may be used in favour of the view that KIC does not stimulate insulin secretion by elevation of NADH-levels by mediation of KIC-dehydrogenase. Moreover the latter experiment demonstrates that an increase of NAD(P)H-fluorescence is no obligatory reaction accompanying the stimulation of insulin release.

The presented results support the view that KIC itself triggered insulin release. The great potency of KIC was not maintained when hydrogen atoms, a hydroxy group or an amino group replaced the α-keto group (Table 1). Moreover the stimulatory activity depended on the structure of the carbon chain of KIC, since α-ketoisovaleric acid released only very little insulin (Table 1). However, it remains unsettled, whether KIC acted on the same receptor site for insulin release as L-leucine and (-)-b-BCH. Information on this point may be provided by a search for competitive inhibitors of insulin release stimulated by those structurally related substances.

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References

1. Panten, U., Kriegstein, E. v., Poser, W., Schönborn, J., Hasselblatt, A.: Effects of L-leucine and α-ketoisocaproic acid upon insulin secretion and metabolism of isolated pancreatic islets. FEBS Letters **20**, 225–228 (1972)
2. Panten, U., Kriegstein, E. v., Poser, W., Schönborn, J., Hasselblatt, A.: Effect of leucine and its metabolites on the function of isolated pancreatic islets. Proceedings of the 7th meeting of the European Association for the Study of Diabetes. Diabetologia **8**, 62 (1972)
3. Panten, U., Kriegstein, E. v., Poser, W., Schönborn, J., Hasselblatt, A.: Wirkung von Leucin und seinen Abbauprodukten auf Insulinsekretion und Stoffwechsel isolierter Pankreasinseln. Proceedings of the 7th Karlsburg Symposium on Problems of Diabetes, Karlsburg 1971
4. Hellerström, C.: A method for the microdissection of intact pancreatic islets of mammals. Acta Endocr. **45**, 122–132 (1964)
5. Panten, U., dal Ri, H., Poser, W., Hasselblatt, A.: Eine Methode der Gewebsumströmung für Fluoreszenzmessungen. Pflügers Arch. ges. Physiol. **323**, 86–90 (1971)
6. Zaharko, D.S., Beck, L.V.: Studies of a simplified plasma insulin immunoassay using cellulose powder. Diabetes **17**, 444–457 (1968)
7. Hellman, B., Idahl, L.-Å., Danielsson, Å.: Adenosine triphosphate levels of mammalian pancreatic B cells after stimulation with glucose and hypoglycemic sulfonylureas. Diabetes **18**, 509–516 (1969)

8. Hellman, B., Sehlin, J., Täljedal, I.-B.: Effects of glucose and other modifiers of insulin release on the oxidative metabolism of amino acids in micro-dissected pancreatic islets. Biochem. J. **123**, 513–521 (1971)
9. Ashcroft, S.J.H., Hedeskov, C.J., Randle, P.J.: Glucose metabolism in mouse pancreatic islets. Biochem. J. **118**, 143–154 (1970)
10. Malaisse-Lagae, F., Brisson, G.R., Malaisse, W.J.: The stimulus-secretion coupling of glucose-induced insulin release. VI. Analogy between the insulinotropic mechanisms of sugars and amino acids. Horm. Metab. Res. **3**, 374–378 (1971)
11. Panten, U., Christians, J.: Effects of 2-endo-amino-norbornane-2-carboxylic acid upon insulin secretion and fluorescence of reduced pyridine nucleotides of isolated perifused pancreatic islets. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmacol. **276**, 55–62 (1973)
12. Fajans, S.S., Floyd, Jr., J.C., Knopf, R.F., Pek, S., Weissman, P., Conn, J.W.: Amino acids and insulin release in vivo. Israel J. med. Sci. 8, 233–243 (1972)

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