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[¹⁴C]Formate Labelling of Bases of Nucleic Acids in Respiring Slices of Rat Tissues

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Alterations in the physiological status of small laboratory animals (e.g. by hormone administration, or by exposing them to a cold environment) frequently cause well-defined changes in the subsequent metabolism of tissue slices taken from certain organs. As part of a general study of such effects, it was of interest to investigate the labelling of nucleic acids in tissue slices. Because of the importance of formate as a precursor of purines (reviewed by Brown, Roll & Weinfeld, 1952; Buchanan, 1952; Elwyn, 1953; Schulman, 1954), it was decided to investigate the possible incorporation of [¹⁴C]formate into the bases of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in respiring slices.

Strangely enough, although there is a vast literature on the incorporation of small molecules into proteins, fatty acids and cholesterol in tissue slices, the nucleic acids have been studied less intensively. Much work has been done, however, on the formation of hypoxanthine in pigeon-liver preparations (summarized by Schulman, 1954).

Labelled inorganic phosphate is incorporated into the RNA of respiring slices of brain (DeLuca, Rossiter & Strickland, 1953) and of pancreas (Hokin & Hokin, 1954). In experiments of this type, most RNA preparations are contaminated with small amounts of highly radioactive phos-

phorus-containing compounds. For this reason, an unequivocal incorporation of the label into RNA had to await the development of adequate analytical methods (Davidson, Frazer & Hutchinson, 1951; Davidson & Smellie, 1952; DeLuca *et al.* 1953). With ¹⁴C-labelled precursors, the earlier experiments were more convincing. Abrams, Goldinger & Barron (1950) showed that both RNA and DNA of bone-marrow slices were labelled from [*carboxy*-¹⁴C]acetate. However, since Bernstein (1952) reported that [*carboxy*-¹⁴C]acetate was incorporated into the ribose of RNA, it is not certain whether the bases or the sugars of the nucleic acids were labelled in these experiments. Reichard & Bergstrom (1951) showed that [¹⁵N]glycine was incorporated into the purines of RNA and DNA of slices of regenerating liver, and, using a similar preparation, Lagerkvist, Reichard & Ehrensvald (1951) and Reichard & Lagerkvist (1953) showed that labelled aspartic acid was incorporated into orotic acid and RNA pyrimidines. LePage (1953) reported that the purines of RNA and DNA were labelled from [α -¹⁴C]glycine in liver and tumour preparations. Using bone-marrow slices, Abrams & Goldinger (1951) found that [*carboxy*-¹⁴C]glycine and [¹⁴C]formate (Abrams & Goldinger, 1952) were incorporated into the purines of both RNA and DNA. Totter (1954) showed that, in addition to the

purines, the methyl group in the 5-position of DNA thymine was also strongly labelled. As far as the present authors are aware, tissue-slice studies with [^{14}C]formate have been confined to bone marrow.

METHODS

Either male rats of the Sprague-Dawley strain or white guinea pigs were used. The animal was killed by decapitation. The liver, while still in position, was perfused with cold 0.154M-NaCl through the hepatic vein. It was then removed, blotted with filter paper and cooled on ice. When required, other tissues were dissected out, blotted free from excess of blood and cooled on ice. Slices (total weight 400 mg. wet wt./flask) were prepared free-hand and placed directly into tared Warburg vessels containing 4.5 ml. saline buffered at pH 7.4 (either Krebs-Ringer bicarbonate or Krebs-Ringer phosphate solution). Each flask was then reweighed and sufficient sodium [^{14}C]formate dissolved in 500 μl . buffered Ringer solution was added to give 6.75 μmoles and 15×10^6 counts/min. per flask. In experiments with inhibitors, the inhibitor was made up in buffered Ringer solution. The final volume was maintained at 5 ml. The flasks were then gassed with the appropriate gas mixture [$\text{O}_2 + \text{CO}_2$ (95:5) for bicarbonate buffer, O_2 for phosphate buffer]. In anaerobic experiments N_2 replaced O_2 , and the gas mixture was passed over heated Cu filings to remove the last traces of O_2 . The vessels were then incubated at $37.4 \pm 0.1^\circ$ for 4 hr., with shaking to ensure proper oxygenation.

At the end of the incubation period the suspending medium was decanted and the tissue was transferred, with the addition of small portions of cold 95% (v/v) ethanol, to a glass tube and homogenized with an electrically driven pestle. The homogenate was centrifuged and the supernatant discarded. Further lipid was extracted from the residue by successive treatment with 5 ml. portions of cold ethanol (twice), 3:1 (v/v) ethanol-ether mixture heated to boiling for 3 min. (three times), and finally ether at room temperature. The residue was extracted at $2-4^\circ$ with 10% (w/v) trichloroacetic acid (TCA), followed by two further extractions with 10% TCA containing carrier formate (0.05M-H. CO_2Na). For the second and third extractions, the tissue residue was suspended in 2.5 ml. distilled water and N-NaOH was added drop by drop until the precipitate dissolved. To this solution was added 2.5 ml. 20% TCA-carrier formate mixture. The precipitate was centrifuged and the supernatant poured off. The residue was finally extracted with 10% TCA containing no carrier formate. This latter extract was free from radioactivity.

Nucleic acids were extracted from the residue by the method of Hammarsten (1947). The dried nucleic acid precipitate was weighed out in small test tubes and hydrolysed with 0.3N-KOH at 37° for 18 hr., 50 μl . alkali per mg. nucleic acid being used. The nucleic acids were separated into DNA and RNA nucleotides by the method of Schmidt & Thannhauser (1945). The purine and pyrimidine bases of both DNA and RNA were liberated by hydrolysis with 72% (w/v) HClO_4 , as described by Marshak & Vogel (1951). The bases were isolated by the method of paper chromatography described by Wyatt (1951). Spots were located by viewing the dried chromatogram in ultraviolet light (Mineralight) against a fluorescent background. The bases were identified by their positions relative to those of

a mixture of authentic samples of purines and pyrimidines. The spots were outlined in pencil and a circular area of filter paper 3 cm. in diameter was punched out from the centre of each of the spots. Each of the paper circles was weighed so that a correction could be made for absorption of β -particles due to the filter paper, as described by LePage & Heidelberger (1951). The radioactivity was determined by placing the paper circle in a gas-flow internal counter (Nuclear Instrument and Chemical Corp., Chicago). The circle was then cut into small pieces and eluted for at least 3 hr. in a large test tube with 10 ml. 0.1N-HCl. The ultraviolet absorption of the eluted base was determined at λ_{max} by means of a Beckman model DU spectrophotometer. The concentration of base in the eluate was calculated from the molecular extinction coefficients given in Table 1. The values found for λ_{max} agreed well with the values quoted in the literature. The specific activity, in terms of counts/min./ μmole , was calculated for each base.

Table 1. Absorption data for nucleic acid bases

Base	λ_{max}	ϵ	Reference
Adenine	265	13 100	Loring, Fairley, Bortner & Seagran (1952)
Guanine	250	11 000	Loring <i>et al.</i> (1952)
Uracil	260	8 130	Ploeser & Loring (1949)
Cytosine	275	10 140	Ploeser & Loring (1949)
Thymine	265	7 940	Vischer & Chargaff (1948)

RESULTS

Labelling of RNA purines of guinea-pig and rat liver

Table 2 shows that the radioactivity of the labelled formate was incorporated into both the adenine and the guanine of the RNA of liver slices from both guinea pig and rat. In these experiments the slices were incubated for 4 hr. in Krebs-Ringer bicarbonate solution buffered at pH 7.4. The table also shows that for rat liver the ratio of the specific activity of adenine to that of guanine was much greater than that observed for guinea-pig liver.

Table 2. Labelling of adenine and guanine in RNA of slices of guinea-pig and rat liver incubated with [^{14}C]formate in Krebs-Ringer bicarbonate solution

	No. of animals	Specific activity (counts/min./ μmole . Mean $\pm$ S.E.M.)		Ratio, adenine/ guanine
		Adenine	Guanine	
Guinea pig	4	1750 $\pm$ 90	1050 $\pm$ 34	1.7
Rat	6	3400 $\pm$ 240	540 $\pm$ 100	6.3

The experiments reported in Table 3 show that at pH 7.4 the RNA purines of rat-liver slices were labelled from [^{14}C]formate in a medium buffered either with bicarbonate or with phosphate. With phosphate buffer the incorporation of the 'label' into adenine appeared to be slightly less than with

bicarbonate, but the incorporation into guanine was greater. Table 3 also illustrates the degree of reproducibility obtained in experiments of this type when duplicate samples were taken from the same liver.

Table 3. *Effect of buffer on the labelling of adenine and guanine in RNA of slices of rat liver incubated with [14 C]formate in Krebs-Ringer solution at pH 7.4.*

Expt. no.	Specific activity (counts/min./ μ mole)			
	Adenine		Guanine	
	Bicarbonate buffer	Phosphate buffer	Bicarbonate buffer	Phosphate buffer
1	3200	2300	470	620
	2700	2300	420	590
2	4500	3200	800	970
	4300	3300	870	1070

Fig. 1. *Effect of pH on the labelling of adenine and guanine in RNA of slices of rat liver incubated with [14 C]formate in Krebs-Ringer solution.*

By using phosphate buffer, the effect of the pH of the incubating medium was investigated for the RNA purines of rat-liver slices (Fig. 1). The extent of the labelling of both purines increased with increasing pH, up to 7.5. Beyond this the effect of pH was less marked. Good labelling was still observed at pH 8.5. The effect of changing the pH was greater for adenine than for guanine. When the pH was less than 6.5, the labelling of the two purines was similar, but at higher pH values more of the label was incorporated into adenine. In many of the subsequent experiments phosphate buffer was used at pH 7.8.

The high optimum pH for the labelling of the RNA purines in rat-liver slices is in contrast to the

low pH of 6.5, reported by Curran (1953) to be optimum for the labelling of cholesterol and fatty acid from [14 C]acetate in similar preparations. That such a low pH gives optimum cholesterol and fatty acid labelling has been confirmed in this Laboratory.

Fig. 2 shows the time course of the labelling of RNA purines of rat-liver slices. After an initial lag period, the incorporation was linear for 2 hr., after which there appeared to be a slight falling-off. At the end of 4 hr. the ratio of the activity of adenine to that of guanine was greater than the ratio found at the end of 2 hr.

Fig. 2. *Time course of the labelling of adenine and guanine in RNA of slices of rat liver incubated with [14 C]formate in Krebs-Ringer solution.*

Table 4. *Effect of metabolic inhibitors on the labelling of adenine and guanine in RNA of slices of guinea-pig liver incubated with [14 C]formate in Krebs-Ringer bicarbonate solution at pH 7.4*

Expt. no.	Inhibitor added	Specific activity (counts/min./ μ mole)	
		Adenine	Guanine
1	None	1010	580
	None (zero-time sample*)	0	0
2	None	1900	780
	KCN (0.03M)	0	0
3	None	1600	1300
	2:4-Dinitrophenol (10^{-4} M)	180	100
	None (anaerobic incubation)	0	0

* Control experiment without incubation (see text).

Metabolic inhibitors

Table 4 shows the effect of metabolic inhibitors on the labelling of RNA purines of guinea-pig liver slices. In Expt. 1 the zero-time sample represents

slices that were prepared in the usual manner, placed in the medium containing radioactive formate, and then homogenized in ethanol without incubation. A figure of 0 is reported if the counting rate of the isolated base was less than one-half of the background counting rate.

Expt. 2 shows that the incorporation into both purines was abolished in the presence of 0.03M-KCN. Expt. 3 shows that there was no incorporation if the conditions were anerobic and that the incorporation was reduced greatly in the presence of 2:4-dinitrophenol (10^{-4} M).

Other tissues

The labelling of RNA purines was determined in slices from a variety of rat tissues. In the two experiments reported in Table 5 the descending order of activity for both adenine and guanine was: spleen, liver, kidney, pancreas, brain, heart. In general, the order of activity was similar to that reported by Drochmans, Marrian & Brown (1952) and Bendich, Russell & Brown (1953) for the RNA

Labelling of DNA purines and pyrimidines

In certain of the experiments with slices of rat liver (Table 6) or spleen (Table 7), the labelling of the four bases of RNA and the four major bases of DNA was studied. In liver slices (Table 6) RNA adenine was most strongly labelled, followed by DNA adenine. The RNA guanine and DNA guanine were next in order. Cytosine and uracil were less well labelled, but the incorporation into thymine was of the same order as the incorporation into DNA guanine. Each of the RNA purines was more strongly labelled than the corresponding DNA purines, but this difference was much greater for adenine than for guanine.

For spleen slices (Table 7), the incorporations were all much greater. Again, adenine was more strongly labelled than guanine and the RNA purines were more strongly labelled than the DNA purines. Each of the pyrimidines demonstrated definite radioactivity, the activity of thymine being of the same order as that of DNA guanine.

Table 5. *Labelling of adenine and guanine in RNA of slices of rat tissue incubated with [14 C]formate in Krebs-Ringer phosphate solution at pH 7.8*

Tissue	Specific activity (counts/min./ μ mole)				Ratio, adenine/guanine	
	Adenine		Guanine		Rat A	Rat B
	Rat A	Rat B	Rat A	Rat B		
Spleen	26 300	21 900	11 200	11 300	2.3	1.9
Liver	4 900	4 800	1 200	980	4.1	4.9
Kidney	3 000	2 300	590	480	5.1	4.8
Pancreas	820	500	170	120	4.8	4.2
Brain	260	300	60	60	4.3	5.0
Heart	110	55	30	30	3.7	1.8

purines of corresponding tissues after the administration of [14 C]formate. The most notable difference was that, in the *in vivo* experiments, kidney RNA purines were more strongly labelled than those of liver or spleen.

The figures quoted for spleen deserve some comment. In the spleens of the two animals used for the experiments reported in Table 5 and in the spleens of two further animals, used in the experiments reported in Table 7, the incorporation of the radioactivity from formate was of a high order. However, in a number of animals studied subsequently, the labelling of the RNA purines of spleen slices, although still greater than the labelling of the RNA purines of liver slices, was less than that reported in Table 5.

The ratio of the specific activity of adenine to that of guanine was less for rat spleen than for most other rat tissues. In this regard rat spleen resembled guinea-pig liver (Table 2).

Effect of fasting for 24 hr.

In the rat, deprivation of food greatly decreases the labelling of fatty acids (Masoro, Chaikoff, Chernick & Felts, 1950; Medes, Thomas & Weinhouse, 1952) and cholesterol (Tomkins & Chaikoff, 1952) from [14 C]acetate in liver slices. Table 8 shows that a fast of 24 hr. caused no similar change in the labelling of RNA purines from [14 C]formate.

Hypophysectomy

Brady, Lukens & Gurin (1951) reported that hypophysectomy increased the labelling of fatty acids from [14 C]acetate in liver slices and Tomkins, Chaikoff & Bennett (1952) reported a decrease in the labelling of cholesterol. Table 9 shows that removal of the pituitary had no significant effect on the labelling of RNA adenine from [14 C]formate. These experiments were performed 14 days after the removal of the pituitary. Other experiments

Table 6. *Labelling of purines and pyrimidines of RNA and DNA of slices of rat liver incubated with [¹⁴C]formate in Krebs-Ringer phosphate solution at pH 7.8*

Base	Specific activity (counts/min./μmole)					
	RNA		DNA		Ratio, RNA/DNA	
	Rat C	Rat D	Rat C	Rat D	Rat C	Rat D
Adenine	5200	6300	1300	1000	4.0	6.3
Guanine	830	830	710	420	1.2	2.0
Cytosine	190	460	290	530	—	—
Uracil	340	540	—	—	—	—
Thymine	—	—	810	550	—	—

Table 7. *Labelling of purines and pyrimidines of RNA and DNA of slices of rat spleen incubated with [¹⁴C]formate in Krebs-Ringer phosphate solution at pH 7.8*

Base	Specific activity (counts/min./μmole)					
	RNA		DNA		Ratio, RNA/DNA	
	Rat C	Rat D	Rat C	Rat D	Rat C	Rat D
Adenine	25 700	20 100	11 700	6 600	2.2	3.0
Guanine	18 000	15 600	9 500	6 800	1.9	2.3
Cytosine	1 700	1 700	510	400	—	—
Uracil	970	990	—	—	—	—
Thymine	—	—	6 800	5 000	—	—

Table 8. *Effect of fasting for 24 hr. on the labelling of adenine and guanine in RNA of slices of rat liver incubated with [¹⁴C]formate in Krebs-Ringer bicarbonate solution at pH 7.4*

	No. of animals	Specific activity (counts/min./μmole, mean ± S.E.M.)	
		Adenine	Guanine
Fed <i>ad lib.</i>	4	2800 ± 300	610 ± 100
Fasted 24 hr.	6	2700 ± 270	710 ± 70

Table 9. *Effect of hypophysectomy and a sham operation on the labelling of adenine in RNA of slices of rat liver incubated with [¹⁴C]formate in Krebs-Ringer bicarbonate solution at pH 7.4*

The animals were studied fourteen days after operation.

	No. of animals	Specific activity (counts/min./μmole, mean ± S.E.M.)
Normal	6	3300 ± 340
Hypophysectomized	11	4000 ± 330
Sham operation	6	4100 ± 390

showed that 2 days after hypophysectomy there was no significant change in the labelling of RNA adenine.

DISCUSSION

The experiments reported in Table 4 on the inhibitory effect of cyanide and 2:4-dinitrophenol provide good evidence that the labelling of the RNA purines is a metabolic phenomenon and that it is not a simple exchange reaction. The inhibition

caused by 2:4-dinitrophenol (10^{-4} M) and the complete absence of labelling under anaerobic conditions strongly suggest that the incorporation is dependent upon the production of energy-rich compounds by oxidative phosphorylation. A similar situation is encountered in the labelling of the nucleotides of RNA with inorganic ³²P in brain slices (DeLuca *et al.* 1953; Findlay, Rossiter & Strickland, 1953). In this regard, it is of interest to note that Abrams *et al.* (1950) found that the incorporation of [¹⁴C]acetate into the RNA and DNA of bone-marrow slices was inhibited by anaerobic conditions and Goldwasser (1953) found that the incorporation of [¹⁴C]adenine into the RNA of liver slices was inhibited by 3×10^{-3} M-KCN.

The general pattern observed for slices of liver and spleen—good labelling of the purines of RNA, with lesser labelling of the pyrimidines, and good labelling of the purines and thymine of DNA, with lesser labelling of cytosine—is in agreement with the *in vitro* experiments of Totter (1954) for bone marrow. Previously, a similar pattern was reported for various rat tissues after the injection of [¹⁴C]formate (Heinrich & Wilson, 1950; Totter, Volkin & Carter, 1951; Bendich *et al.* 1953). In experiments of this type, the nucleic acid purines were found to be labelled in positions 2 and 8 (Heinrich & Wilson, 1950; Drysdale, Plaut & Lardy, 1951) and the thymine was labelled in the methyl group at position 5 (Totter *et al.* 1951).

The finding that adenine was labelled more strongly than guanine in RNA is in agreement with the results of *in vitro* studies with bone-marrow

slices, in which either [*carboxy*- ^{14}C]glycine (Abrams & Goldinger, 1951) or [^{14}C]formate (Abrams & Goldinger, 1951; Totter, 1954) was the source of the label. In the experiments reported here with liver slices, the ratio of the activity of RNA adenine to that of RNA guanine was found to depend upon the organ studied (Table 5), the species (Table 2), the nature of the buffer (Table 3), the pH (Fig. 1) and the time of the incubation (Fig. 2). It is not surprising, therefore, that with *in vivo* experiments great differences are reported in the literature for this ratio. In such experiments a wide variety of species, tissues, injection times etc. were used.

The finding that for both liver and spleen slices the labelling of RNA adenine and guanine was greater than that of the corresponding DNA purine is similar to previous *in vivo* experiments, in which liver and spleen were studied after the administration of [^{14}C]formate (Bendich *et al.* 1953; Smellie, McIndoe & Davidson, 1953). *In vivo* experiments with ^{32}P , in which animals were killed at short intervals after administration of the isotope, showed that the labelling of RNA was considerably greater than the labelling of DNA, for tissues not rapidly proliferating (Brues, Tracy & Cohn, 1944; Hammarsten & Hevesy, 1946; Volkin & Carter, 1951; Davidson & Smellie, 1952). In tissue-slice experiments with ^{32}P , RNA is much more strongly labelled than DNA. Indeed, because of the possibility that non-radioactive DNA may be contaminated with traces of much more radioactive RNA, *in vitro* studies with ^{32}P , such as those of DeLuca *et al.* (1953) with brain slices and Kit, Bacila & Barron (1954) with lymphocytic cells, make it uncertain whether DNA is labelled at all. This would be in conformity with the suggestion that ^{32}P is incorporated into DNA only during cell division (Marshak, 1941; Hull & Kirk, 1950; Osgood, Li, Tivey, Duerst & Seaman, 1951; Stevens, Daoust & Leblond, 1953).

The figures reported in Tables 6 and 7 indicate that, unlike *in vitro* studies with ^{32}P , DNA was strongly labelled when the radioactive precursor was [^{14}C]formate. If it were assumed that the DNA purines were not labelled at all and that the radioactivity observed was due to contamination of non-radioactive DNA with radioactive RNA, the contamination would have to be gross (more than 50% in some instances). Such gross contamination would be detected by the presence of uracil in the chromatograms. Also, the labelling of thymine could not be explained in terms of contamination of the DNA with RNA. The *in vitro* labelling of DNA purines in bone-marrow slices from [^{14}C]acetate (Abrams *et al.* 1950), [^{14}C]glycine (Abrams & Goldinger, 1951) and [^{14}C]formate (Abrams & Goldinger, 1952; Totter, 1954) has been reported.

Alteration of the physiological status of the animal would appear to have little effect on the subsequent ability of liver slices to label RNA purines. This is illustrated by the findings that food deprivation for 24 hr. (Table 8) or removal of the pituitary gland (Table 9) caused no significant change. In addition, it was found that the presence of insulin *in vitro*, either with or without the addition of glucose, had no significant effect on the labelling of RNA purines in rat-liver slices (unpublished experiments). Exposing the animals to an environmental temperature of 3° for periods of time ranging from 3 hr. to 28 days, or exposing them for 3 hr. to temperatures ranging from 5° to -10°, also had no consistent significant effect on the labelling of the RNA or DNA purines of liver or spleen slices. These findings are in sharp contrast to the labelling of cholesterol (Tomkins & Chaikoff, 1952; Tomkins *et al.* 1952), fatty acid (Masoro *et al.* 1950; Brady *et al.* 1951; Medes *et al.* 1952) and phospholipid (Rossiter & Kline, 1955) of liver slices from [^{14}C]acetate. The degree of labelling of these substances is influenced greatly by the preceding physiological condition of the animal.

SUMMARY

1. When slices of rat liver or spleen were allowed to respire in Krebs-Ringer solution containing [^{14}C]formate, radioactivity was detected in the adenine and guanine of RNA and the adenine, guanine and thymine of DNA. The labelling of the RNA purines of liver took place when the slices were incubated in either phosphate or bicarbonate buffer. Optimum labelling occurred at a pH greater than 7.5.

2. Under similar conditions, the purines of the RNA of slices of guinea-pig liver were labelled. The labelling was inhibited by KCN (0.03M), 2:4-dinitrophenol (10^{-4}M), or by rendering the conditions anaerobic.

3. Adenine and guanine of RNA in slices of other organs were also labelled. For the rat, the descending order of activity was: spleen, liver, kidney, pancreas, brain, heart.

4. Labelling of the purines of RNA was not affected by a previous fast of 24 hr. or by removing the pituitary gland.

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Formation and Properties of the Activator of Plasminogen and of Human and Bovine Plasmin

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Plasminogen is the precursor of plasmin, a proteolytic and fibrinolytic enzyme of blood. Streptokinase, an agent produced by certain streptococci (Garner & Tillett, 1934), effects the conversion of human plasminogen into plasmin (Kaplan, 1944; Christensen, 1945), presumably by a direct enzymic effect (Christensen & MacLeod, 1945). Bovine plasminogen was activated only very slightly by streptokinase, but large amounts of plasmin were

produced in bovine plasminogen by a tissue activator (Astrup & Permin, 1948), probably by a stoichiometric reaction (Astrup, 1951). Geiger (1952) demonstrated an increased proteolytic activity in mixtures of globulin preparations from man and other species after activation by streptokinase. He suggested that a complement-like factor was involved in the activation of plasminogen by streptokinase. Müllertz & Lassen (1953)