COMMUNICATIONS

STUDIES ON DEVELOPING RETINAL VESSELS

V. MECHANISM OF VASO-OBLITERATION

(A Preliminary Report)

BY

NORMAN ASHTON, CLIVE GRAYMORE, AND
CHRISTOPHER PEDLER

Department of Pathology, Institute of Ophthalmology, University of London

SINCE the discovery of the specific effect of oxygen on the immature retina, which manifests itself in vasoconstriction leading to total obliteration of the ingrowing vascular complexes (Ashton, Ward, and Serpell, 1953, 1954), the work of this department has been concerned with experiments designed to elucidate the mechanism of this effect. The evidence that the phenomenon is entirely confined to vessels actually growing in the retina, and then only when the retina is immature and in its normal anatomical position, has been recently reviewed elsewhere (Ashton, 1957). Broadly speaking there would appear to be three main theoretical explanations for the closure of these vessels:

- (1) That hyperoxia in the retina leads to the elaboration of a vasoconstrictor substance;
- (2) That retinal hyperoxia leads to the removal of a vasodilator substance essential to the patency of the vessels;
- (3) That the vessels close from external pressure.

Consideration has already been given to the first possibility that hyper-oxygenation of retinal tissue might produce a vasoconstricting metabolite, which gradually accumulates until the retinal arterioles close in spasm, to be followed by passive collapse of the venous side of the circulation, and it was suggested that the specific action of this hypothetical factor might be related to the immature nerve supply of growing retinal vessels (Ashton and others, 1954).

It is difficult to imagine, however, upon what cells in such a primitive vascular system such a metabolite could act, especially since we have found that known vasoconstrictors do not obliterate growing retinal vessels, and that sympathetic innervation is not directly or indirectly concerned in the mechanism of oxygen vaso-obliteration (Patz, 1955; Cook and Ashton, 1955). Furthermore, we have found that extracts of retinae in which vaso-obliteration had been induced (extracts prepared in high ambient concentrations of oxygen) have no effect when injected *via* the vitreous on to

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normal retinae in direct-observation experiments on the living animal. Nor did such extracts show any evidence of a smooth muscle stimulant as tested against preparations of rat colon. The details of this experiment were as follows:

Experiment No. 173

(1) Two kittens were placed with their mother into an ambient oxygen concentration of 70–80 per cent. for 3 days to ensure total retinal vaso-obliteration.

(2) The two animals were then immediately transferred to a large perspex chamber filled by an atmosphere containing 90 per cent. oxygen, and fitted with sealed armholes. They were then killed with Nembutal, and all four retinae were removed, homogenized in distilled water, and filtered through a sintered glass filter of 28μ average pore size, giving an approximate concentration of 40 mg./ml. The extract was then divided into two parts, one to be used for Stage (3) and the other for Stage (4).

(3) As soon as the extraction stage was complete, a "limbal window" was inserted into the eye of a normal 10-day-old kitten. The animal was then transferred to the apparatus for direct retinal observation (Pedler, 1957), and left until the retinal vasculature was fully open. 0.1 ml. of the extract from Stage (2) was then injected with a fine-bore needle through the vitreous onto the retinal surface (Fig. 1). No effect was seen after an observation period of 60 min. This dosage was repeated with similarly negative results, and finally 0.1 ml. of the extract was given intravenously, again with no visible effect on the retinal vessels.

(4) The remaining extract was handed to Dr. N. Ambache, who found no evidence of a smooth muscle stimulant as tested against a preparation of rat colon. This experiment was repeated with an extract from a further four oxygenated retinae, again with negative results (Exp. 205).

While it is realized that these findings by no means exclude the possibility that the closure of vessels may be due to active vasoconstriction, the negative evidence so far obtained, directly or by inference, discourages the concept.

The second possibility, that vessel closure is due to the removal of a vasodilator substance inherent in the vasoformative process, has also been discussed previously (Ashton and others, 1954). It is noteworthy, however, that for growing vessels to be obliterated by hyperoxia it is essential for them to be actually within the retinal tissue; developing vessels within the vitreous, on the iris, or in the cornea are not affected in this way. If oxygen produced vaso-obliteration merely by neutralizing a vasoformative substance, it is difficult to understand why it should be so remarkably selective, although we have suggested that this might be attributable to the peculiar anatomical relationship between the retina and choroid. It is a difficult question to investigate, however, and we have no conclusive evidence either to deny or to support the possibility.

The third possibility, that the vessels may close through external pressure, had been overlooked in our previous discussion on the mechanism of vaso-obliteration, but it became apparent as our original hypotheses seemed

inadequate to explain all the observed facts. An increase of intra-ocular pressure as a result of hyperoxia can be excluded as a cause of the oxygen effect: first because compression of the globe does not produce the picture of vaso-obliteration (Ashton and Pedler, 1957) and secondly because oxygen vaso-obliteration has been observed in the opened eye. But could the process of vaso-obliteration be partly or wholly due to swelling of the cells or tissues surrounding the blood stream?

Although it is known that endothelial cells have the property of swelling when subjected to irritating stimuli (Krogh, 1922), we can probably exonerate them here as oxygen vaso-obliteration does not occur in growing vessels outside the retina. Swelling of the retinal tissue, however, remains a possibility, and in considering one of the ways in which this might theoretically occur we were led to some new and interesting findings.

The hypothesis upon which our experiments were based was as follows. Until recently it has been the orthodox view that the cells of the body are in osmotic equilibrium with their extracellular environment; that is, their contents are isotonic with the blood plasma. It would now appear that this is the exception rather than the rule (Bartley, Davies, and Krebs, 1954); in fact, it has been estimated that the osmotic pressure of the cell fluids is normally 50 to 100 per cent. greater than that of the extracellular fluid (Robinson, 1950). If these findings are substantiated, the idea of the osmotic equilibrium being governed solely by the physical properties of cell membranes would no longer be tenable, and it would be necessary to postulate that the water content of the cell, and therefore the cell size, is dependent upon some dynamic process involving a continuous supply of energy.

That this concept of osmo-regulation may be correct is suggested, for instance, by the ability of some cells of the renal tubules to maintain a normal internal environment despite the variable tonicity of the urine which bathes them. In fact, this consideration led Robinson (1950) to investigate the behaviour of tissue slices cut from the kidneys of normal adult rats. By measuring the oxygen consumption and the amount of water in the tissues under varying conditions, he found that respiration was in fact more important than the osmotic pressure of the external media in determining the amount of water in the cells. When respiration was inhibited by cyanide, water passed into the cells. In short, the energy for the transport of water across the cell membrane was in this case derived from cell respiration.

It is interesting to note in his experiments that the imbibition of water was a rapid process, being almost complete in 2 minutes and then maintained for several hours. It has been suggested that this steady-state exchange depends on energy-driven "pumps" located in the mitochondria, as these structures when isolated from the cell can be shown to do osmotic work by moving substances against concentration gradients (Bartley and others, 1954).

Although there is not complete agreement on the existence of these pumping mechanisms, it having been denied that the cell is significantly

hypertonic and claimed that cellular swelling is isosmotic in nature (Mudge, 1956), the cardinal point that tissue cells swell when placed in an environment where the metabolism is inhibited is not in question. Some years ago, Trowell (1946) showed that liver cells when subjected to anoxia increased in size because of the passage of water into the cells. This process he described as "intracellular oedema", although in this instance he did not attribute it to osmotic absorption.

Returning now to the retina, it will be recalled that it has the highest rate of respiration of any tissue (Warburg, 1927) and a higher rate of glycolysis in air than most other tissues. As early as 1924 it had been suggested, by Warburg, Posener, and Negelein, that the various layers of the retina possessed their own peculiar metabolism, some of the cells being responsible for the high glycolytic activity, while the others were almost completely oxidative in character. This suggestion was later strongly supported by other workers (Sjöstrand, 1953; Strominger and Lowry, 1955; Lowry, Roberts, and Lewis, 1956).

In both monkey and rabbit it would appear that the rod and cone layer is predominantly concerned with oxidative metabolism, while the inner layers, especially of avascular retinæ as in the rabbit, are predominantly glycolytic in activity. The immature retina, at a stage when oxygen vaso-obliteration may be obtained, differs from the mature retina in the important respect that retinal vascularization is still developing, so that it is reasonable to suppose that the metabolism of the inner layers of such a retina may be predominantly glycolytic. Indeed, we have already deduced from numerous experiments that the immature retina probably has a metabolism differing from that in the mature retina.

If such is the case, it would follow that fluid exchanges through the walls of the inner retinal cells—and, therefore, the cell-size—might be dependent upon energy derived, not from respiration but from glycolysis. With these considerations in mind, we argued that, if swelling of the cells is an important factor in the obliteration of retinal vessels by oxygen, then the inhibition of glycolysis by known enzyme poisons might also lead to vaso-obliteration.

We have therefore investigated the action of two well-known inhibitors of glycolysis.

(1) *Sodium Fluoride*.—The *in vitro* fluoride inhibition of glycolysis has been demonstrated repeatedly in many tissues. The normal phosphorylating glycolytic route is thought to occur in the retina, and it has been confirmed that this system is particularly sensitive to fluoride (Dickens and Greville, 1932), which interrupts the glycolytic sequence at an early stage, and inhibits the production of lactic acid (Kerly and Bourne, 1940). Both phosphoglucomutase (Najjar, 1948) and enolase (Warburg and Christian, 1942) are inhibited by fluoride, presumably by the effective removal of essential Mg ions by complexing with fluoride and phosphate.

(2) *Sodium Iodoacetate*.—This is a general poison for SH enzymes and thus has a rather wide and non-specific effect. It is a strong glycolytic inhibitor, irreversibly inhibiting phosphoglyceraldehyde dehydrogenase in low concentrations (Rapkine, 1938). This is one of the important energy “trapping” stages in glycolysis, and as the other important step, namely, that catalysed by enolase, occurs further down in the metabolic sequence, the effect of interrupting glycolysis at the phosphoglyceraldehyde stage is obvious. Glycolysis will cease to be an effective source of energy. As in the case of fluoride, iodoacetate inhibits the production of lactic acid in the retina; it produces a characteristic rod cell degeneration, which has been attributed to glycolytic inhibition (Noell, 1952).

I. SODIUM FLUORIDE EXPERIMENTS

Two solutions of sodium fluoride were prepared, the first isotonic with normal blood at 0.61 gm./100 ml., and the second a hypertonic saturated solution. Glass-distilled water was used and the pH adjusted to 7.1. The figure for isotonicity was obtained both by calculation and by direct test on the kitten's red blood cells, crenation and swelling being taken to indicate hyper- and hypotonicity respectively.

The sodium fluoride was injected through the sclera onto the retinae of kittens in which limbal windows had been inserted. The effect on the retinal vessels was then studied by direct observation. The details were as follows:

A very fine-bore needle (“Record” 3/10 mm. $\frac{1}{2}$ in.), attached to a 1-ml. tuberculin syringe, was gently inserted through the sclera just posterior to the rim of the limbal window, and its tip guided towards the retinal surface. When the position of the needle was satisfactory, the fluid was injected so that it remained in the form of a localized bleb just within the confines of the vitreous (Fig. 1). In different experiments, doses varying between 0.1 and 0.5 ml. were given.

Only one intravenous injection of isotonic fluoride was administered, and in this experiment 4.2 ml. were injected.

A remarkably consistent series of events was observed in nearly all the experiments with sodium fluoride; these will be described below in the order in which they occurred and reference to Table I (overleaf) will show the exact times after injection when the changes first appeared and when they were fully developed.

FIG. 1.—Diagram showing method used for injecting the vitreous in direct-observation experiments on the retinal vessels. The anterior ocular structures have been replaced with a limbal window.

TABLE I
SODIUM FLUORIDE EXPERIMENTS

Experiment No.	Animal No.	Age (days)	Weight (g.)	Eye	Dose (ml. 0.61 per cent. solution)	Closure Started (min.)	Closure Maximum (min.)
177	1	11	300	L	0.2	5	18
	2	12	290	L ₍₁₎	0.1	3	10
				L ₍₂₎	0.1	3	28
186	1	9	260	L	0.5	5	18
188	1	9	230	L	0.3	3	12
192	1	6	210	R	0.2	2	22
193	1	6	210	L	0.2	3	20
194	1	7	215	L	0.2	1	23
195	1	7	195	R	0.2	3	20
197	1	14	230	R	0.2	1	5

The Table shows the time taken for retinal vaso-obliteration to appear and to develop fully after the intravitreal injection of isotonic sodium fluoride in ten experiments.

(1) Sequence of Changes

The first change in the retinal vasculature occurred a few minutes after the administration of the isotonic fluoride and consisted of a rapid and marked venous distension. This occurred primarily in the three main vessels, but quickly spread to involve the branch veins and venules until the whole of the venous side of the circulation became grossly distended. Those experiments where the optic disc was clearly visible also showed that simultaneously with the appearance of venous distension there occurred an apparent swelling of the optic disc with nipping of the veins as they entered its periphery (Fig. 2, opposite). Soon after the venous engorgement became apparent the precapillary arterioles showed a progressive narrowing, until finally they disappeared altogether. The main arteries were the next to close, and these did so in the same manner as the smaller branches, completely disappearing from view. During the period in which the arterial closure was maximal, a milky-white reflex developed within the substance of the retina. Meanwhile the blood flow in the veins became progressively slower, until in the majority of experiments a pulsatile oscillation appeared of the same frequency as that of the arterial pulse, and no forward movement of the blood column could be seen. When 20 minutes had elapsed after injection, it was usual to find an almost completely avascular retina surrounding the point of injection, presenting perhaps one or two dilated veins and isolated patches of capillaries showing complete stasis. In one exceptional case (Exp. 197), complete obliteration occurred in 5 minutes.

FIG. 2.—Retina showing the fluoride effect on the retinal vessels in the disc region. The disc and hyaloid artery may be seen on the extreme left of the picture. Note that the optic nerve fibres stand out distinctly, and the arteries and capillaries are largely obliterated, while the vein is markedly engorged and constricted as it enters the disc. $\times 60$.

Finally, the fragmented stagnant blood columns in the remaining veins were replaced by a clear fluid containing only a few red cells. This apparent haemodilution coincided as a rule with the cessation of the oscillation previously mentioned, and with the resumption of flow in the normal direction.

In summary, 0.2 ml. isotonic sodium fluoride when injected into the vitreous will consistently produce complete or almost complete vaso-obliteration in the retina within an average period of 20 minutes. Similar, and also larger, quantities of sodium fluoride instilled onto the vessels of the conjunctiva and onto the vessels of the exposed omentum were without effect.

Control Experiments, using saline solution and distilled water under exactly similar experimental conditions, produced no vaso-obliteration whatever, thus effectively demonstrating that the vascular closure resulted from the introduction of sodium fluoride only, and not, for example, from the injection of a cold fluid or indeed from raising the intra-ocular pressure. In one experiment, in order to be certain that the injected fluoride actually reached the surface of the retina, the solution was coloured with aqueous methylene blue. Immediately after administration, this could be seen lying on the retina in the form of a localized bleb of colour, which slowly became diluted, and diffused evenly outwards over the retinal surface.

The changes which have been described were usually localized to the area immediately surrounding the point of injection, so that the vessels elsewhere in the retina were affected only after a long period if at all. In several experiments (Exps 177, 188, 193, and 194), the retinae were observed for some hours after total vaso-obliteration. In these it was noted that a considerable degree of vessel re-opening occurred, mainly in the reverse direction to that

of the obliterative sequence, that is to say, the veins were the first to re-open and the main arteries the last. The actual time after injection at which the vessels started to re-appear was variable, but on the average some re-opening was visible after 30 minutes.

It was mentioned above that the veins which did not fully close were filled by a clear fluid during the period of maximum vaso-obliteration. Once it was seen that re-opening had begun, this clear fluid was gradually replaced by blood until the vessels had once again attained a fairly normal appearance. Those veins which were fully closed, however, re-opened and filled with blood, but contained no normal blood flow, suggesting that there was still a central block in operation.

Apart from the actual vaso-obliteration, one of the most striking observations made during this series of experiments was that after fluoride injection the retina appeared to swell up on each side of the obliterated vessels (Fig. 3) so that, instead of lying superficially, they appeared to recede deeply within its substance. This appearance gradually became more marked as the experiment progressed, until the retina eventually detached from the choroid, and this appeared to occur more readily in those areas where maximum vessel closure was obtained. As soon as this occurred, there was a complete re-opening of all the vessels overlying the detached area, even though the attached portions showed complete vaso-obliteration.

FIG. 3.—In the sodium fluoride effect, obliterating vessels appear to recede deeply into the retinal tissue. The above diagrams (before and after fluoride) show how this might be due to swelling of the retinal tissue.

(2) Effect of Intravitreal Injection of Hypertonic Solutions

In four direct-observation experiments, hypertonic solutions were injected into the vitreous after complete vaso-obliteration had been induced by isotonic sodium fluoride. In the first two (Exp. 177), 30 per cent. sodium chloride was used, and in the others (Exp. 186) 18 per cent. sucrose in distilled water. In all cases, a rapid and complete re-opening of almost the entire vasculature took place. The appearance of these vessels, however, differed from those seen during a spontaneous re-opening after fluoride obliteration in that a functional blood flow was never re-established, the blood column within them appearing as a series of stationary beads, in spite of the fact that the

vessels were widely dilated. These appearances were due to intravascular coagulation.

In two experiments (Exps 186 and 208) where hypertonic sodium fluoride was injected instead of the isotonic solution, the vaso-obliterative effect was less complete and re-opening of the vessels occurred more readily; but these experiments in themselves are inconclusive.

(3) Intravitreal Fluoride with Survival

- In order to compare the fluoride effect with oxygen vaso-obliteration as seen in retinae injected with Indian ink, it was decided to try the effect of intravitreal fluoride in the intact eye. Accordingly 0.1 ml. of isotonic sodium fluoride was injected into the vitreous in three anaesthetized kittens at different intervals, and the animals were then killed by the intraventricular injection of Indian ink, so that we were able to examine the six eyes 15 minutes, 40 minutes, 4 hours, 8 hours, and 24 hours after injection (Exps 179 and 190). When the excised eyes were subsequently opened and examined, it was found somewhat surprisingly that all the vessels were fully open, and that there had apparently been no vaso-obliteration. These findings suggested that either the sodium fluoride had not produced vaso-obliteration, or that the intraventricular injection of Indian ink had re-opened them. An experiment was therefore carried out in order to determine which of these two possibilities was correct.

A limbal window was inserted into the eye of a 6-day-old kitten, and vaso-obliteration was produced by the intravitreal injection of 0.2 ml. isotonic sodium fluoride. Complete vaso-obliteration had been produced 22 minutes after injection. The left side of the chest was then opened, and Indian ink injected through the tip of the left ventricle. It was immediately seen that all the previously closed vessels re-opened at once and completely filled with Indian ink (Exp. 192).

These experiments demonstrated the danger of interpreting Indian ink injection as a measure of vessel closure, and again emphasized that the method should be used rather as a measure of the ability of the vessels to re-open.

(4) Direct Observation and Intravenous Sodium Fluoride

Only one attempt was made to observe the effect on the retinal vessels of intravenous isotonic sodium fluoride; it was found that almost complete vaso-obliteration could eventually be produced in the immature retina by repeated doses (total = 4.4 ml. 0.61 per cent. solution), but only after the animal had received a lethal dose and was at the point of death (Exp. 177).

(5) Intravitreal Fluoride in Adult Cats

In two adult cats, isotonic sodium fluoride was injected into the vitreous under direct-observation, so that any effect upon the vessels could be recorded.

In all cases, neither vasoconstriction nor vaso-obliteration was seen as a result of the injection, even after relatively coarse procedures such as removing the vitreous and replacing it with 0.61 per cent. sodium fluoride solution (Exps 180 and 198).

II. SODIUM IODOACETATE EXPERIMENTS

In four kittens, varying in age between 8 and 10 days, and in weight between 165 and 275 gm., the effect of intravitreal injections of 0.154 molar solutions of iodoacetic acid (neutralized with sodium hydroxide to a pH of 7.2) was noted six times, using the same technique as that described for the sodium fluoride experiments (Exps 201, 202, 206, and 207). In all cases some vessel closure was observed, and in three of them complete vaso-obliteration was produced at the site of injection. In general, the appearance and order of vessel closure was the same as that observed after sodium fluoride injection, the main difference being the rate at which closure appeared. Whereas, in the case of fluoride, maximum obliteration developed about 20 minutes after injection, the closure brought about by sodium iodoacetate began as soon as the dose had been given, and was at its maximum 2 to 4 minutes afterwards (Table II). Similarly, some spontaneous re-opening of the vessels was seen, but again this occurred without significant restoration of the normal blood flow.

TABLE II
SODIUM IODOACETATE EXPERIMENTS

Experiment No.	Animal No.	Age (days)	Weight (g.)	Eye	Dose (ml. 0.154 M solution)	Closure Started (min.)	Closure Maximum (min.)
201	1	10	175	L	0.2	1	2
				R	0.2	1	3
202	1	8	165	L	0.2	1	Animal died
206	1	9	275	L	0.2	<1	1
207	1	10	275	L	0.2	<1	4
				R	0.2	<1	2

The Table shows the time taken for retinal vaso-obliteration to appear and to develop fully after the intravitreal injection of isotonic sodium iodoacetate in six experiments.

CONCLUSIONS

These experiments have therefore shown that oxygen vaso-obliteration can be very closely mimicked by introducing sodium fluoride or iodoacetate into the vitreous. As in the case of oxygen, the effect is confined to developing vessels in the immature retina, and it is similarly reversed by retinal

detachment. It is tempting to believe that the two processes share a common mechanism, but at the present time we have very little evidence that this is so. Nor have we yet shown that the hypothetical considerations which led us to devise these experiments, namely, the inhibition of glycolysis with a breakdown in osmo-regulation, are in fact those operating in the fluoride and iodoacetate effect.

In the fluoride effect, the possibility that the vessels may be obliterated by the passage of fluid into the retina is supported by several observations: separation of the nerve fibres and constriction of the veins at the disc, depression of vessels in the retinal substance, development of a milky-white opacity as the vessels close, and reversal of vascular closure by injecting hypertonic solutions into the vitreous.

There are, however, so many ways by which irritant chemicals, such as sodium fluoride or iodoacetate, might produce these changes that it is not possible without further work to hold their enzyme-inhibitory properties as alone responsible. Furthermore, although their failure to produce obliteration in adult retinal vessels might be taken to imply metabolic differences, other factors, such as variation in the structure of the retina and its vessels or in the blood pressure within the vessels, must be taken into consideration. This is all the more important since we have been unable to reproduce vaso-obliteration in the adult retina even with enzyme poisons, which might be expected to arrest completely retinal metabolism, such as cyanide (Robbie and Leinfelder, 1948), or a combination of cyanide and sodium fluoride, or mercury salts.

By histological methods, intracellular or extracellular oedema in the inner layers of the retina can readily be demonstrated in kittens subjected to oxygen, sodium fluoride, or iodoacetate, but experience has shown that such appearances must be assessed with caution, for it is one of the commonest histological artefacts seen in eye sections. We have found it also in a few control animals, but this does not exclude fluid imbibition as a possible cause of vaso-obliteration, since it is to be expected that cells or tissues dying in fixative might well undergo terminal osmotic changes and present appearances identical with those we are seeking. Similarly, the various processes of dehydration to which histological material is subjected greatly adds to the difficulty in interpreting negative findings.

The problem is clearly a highly complex one, and much more evidence is required before fluid imbibition in the retina can be incriminated as the cause of vaso-obliteration, but this report on the action of sodium fluoride and iodoacetate, together with the considerations which prompted their investigation, are presented now in a preliminary form to show the direction in which our experiments are leading us, and to emphasize the possible role of increased intra-retinal tension in vaso-obliteration, a mechanism which has not previously been considered, either by ourselves or by other workers.

SUMMARY

Some mechanisms which might be involved in the phenomenon of oxygen vaso-obliteration are discussed. On the hypothesis that the vessels might close through external pressure from fluid imbibition into the retina as a result of an interruption in glycolysis, we have investigated the action of sodium fluoride and sodium iodoacetate, which are well-established glycolytic inhibitors.

It was found that both these substances when injected into the vitreous of the living kitten gave rise to total obliteration of the retinal vessels in a manner closely resembling that induced by oxygen exposure. In a similar way, the vaso-obliteration is confined to the immature retina, no effect having been observed in adult cats or in extra-ocular vessels, and, as in the case of oxygen, the vessels re-open when the retina becomes detached from the choroid.

Reasons are given for believing that the fluoride effect may be due to swelling of the retinal tissue around the vessels. However, it is not yet established whether this idea is correct. Nor is it known whether the mechanism of oxygen vaso-obliteration is related to that obtained with sodium fluoride or iodoacetate, but it is reasonable to expect that it may be, and the suggestion is made that future work should further explore this possibility.

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